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Analisi degli effetti citotossici del diclofenac in linee cellulari di melanoma umano

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Introduzione

1.1 Il melanoma

Il melanoma è un tumore molto aggressivo la cui sede elettiva è rappresentata dalla cute ma che in misura minore, può interessare anche la mucosa orale e anogenitale, l'esofago, le meningi e l'occhio. La diagnosi precoce, e la conseguente escissione chirurgica del melanoma, rappresentano la terapia elettiva e in molti casi risolutiva del tumore mentre una diagnosi tardiva, è associata ad una prognosi infausta [1].

Negli ultimi venti anni l'incidenza del melanoma è aumentata di oltre il 4% all'anno in entrambi i sessi, e in particolare il melanoma cutaneo in Italia ha un'incidenza di 14.3 casi per 100.000 uomini e di 13.6 casi per 1000.000 donne (dati AIRTUM). A differenza di molte altre neoplasie il 50% dei melanomi è diagnosticato entro i 60 anni d'età. Il melanoma nel suo stadio metastatico è poco suscettibile alle terapie convenzionali e, la metastasi linfonodale, può associarsi al 70% di mortalità dopo 10 anni [2,3]. La risposta infiammatoria acuta, conseguente all'escissione chirurgica del melanoma primario, può regolare il potenziale metastatico del melanoma. È noto, infatti, che le citochine infiammatorie inducono l'espressione delle integrine e di altre molecole di adesione in linee cellulari di melanoma umano [4].

Il melanoma cutaneo è clinicamente asintomatico e al momento della diagnosi la maggior parte delle lesioni ha diametro superiore a 10 mm; il segno clinico più importante è la modificazione di colore e di forma di una lesione pigmentata. Diversamente dai nevi benigni (non displastici), il melanoma presenta spesso variazioni di pigmentazione che si manifestano con la comparsa di sfumature nerastre, brune, rossastre, bluastre, grigie.

Occasionalmente, si osservano anche aree ipopigmentate biancastre o color "carne viva", dovute a una regressione focale del tumore. I contorni del melanoma non sono lisci, arrotondati e regolari come quelli dei nevi nevocitici, ma piuttosto irregolari e spesso indentati.

La crescita del melanoma può essere radiale e verticale [5]. La crescita radiale è rappresentata dalla capacità del melanoma di svilupparsi orizzontalmente all'interno dell'epidermide e lungo la giunzione dermo-epidermica, spesso per un lungo periodo di tempo. In questa fase di crescita le cellule del melanoma non hanno la capacità di metastatizzare; le varianti specifiche di tale neoplasia comprendono la lentigo maligna (tipicamente insorge sulla cute esposta, in soggetti anziani, e rimane tale per molto tempo, fino a diversi decenni, prima di evolvere in un melanoma con capacità metastatiche), il melanoma a diffusione superficiale (il tipo di melanoma più comune, che solitamente coinvolge la pelle che è esposta al sole) e il melanoma lentigginoso acrale e mucoso (non è correlato con l'esposizione al sole) (Figura 1). Con il tempo, la crescita neoplastica assume un carattere verticale, e il melanoma comincia a crescere nel derma.

Le cellule del melanoma maligno sono solitamente più grandi delle cellule neviche benigne. Esse hanno grossi nuclei con contorni irregolari e cromatina tipicamente addensata alla periferia, attorno alla membrana nucleare e nucleoli rossastri (eosinofili) prominenti. Questi elementi neoplastici crescono in nidi mal definiti, o a singole cellule, a tutti i livelli dell'epidermide nella fase radiale di crescita, mentre nel derma appaiono come grossi aggregati balloniformi. L'estensione (spessore) e le caratteristiche istologiche della componente a crescita verticale determinano il comportamento biologico della neoplasia. L'aspetto delle cellule tumorali è simile sia nella fase di crescita radiale che in quella verticale. Mentre la maggior parte dei nevi e dei melanomi sono distinti abbastanza semplicemente grazie alla valutazione del loro aspetto, esiste una quota minore di lesioni

atipiche che occupano un'area istologica grigia, e che sono state definite tumori melanocitici con potenziale maligno incerto [6,7]. Queste lesioni richiedono un'escissione chirurgica completa e un attento "follow-up" clinico.

I fattori che più predispongono i soggetti all'insorgenza del melanoma sono genetici e ambientali [8]. Il melanoma tende a comparire più frequentemente nelle zone del corpo che sono più esposte al sole. Negli uomini insorge prevalentemente nella parte superiore del dorso, mentre nelle donne l'incidenza è relativamente alta sia nel dorso che nelle gambe. Gli individui di pelle chiara, inoltre, hanno un rischio maggiore di sviluppare questa neoplasia, rispetto ai soggetti con cute ben pigmentata. La relazione tra melanoma ed esposizione al sole non sembra, tuttavia, essere così lineare come accade per altri tipi di tumori della pelle. Il 10-15% dei melanomi presenta familiarità e la maggior parte dei geni associati alla familiarità del melanoma, codificano per proteine che rappresentano dei *tumor suppressor* molto ben caratterizzati, le cui mutazioni sono state identificate anche in alcuni tumori sporadici. Altre varianti geniche, che aumentano lievemente il rischio d'insorgenza di melanoma in popolazione di pelle chiara, regolano la produzione della melanina. Tra queste varianti sono inclusi i geni che codificano per MC1R (recettore della melanocortina 1), ASIP (proteina segnale agouti, che codifica per un regolatore della segnalazione del recettore della melanocortina) e TYR (tirosinasi, enzima specifico dei melanociti, necassario per la sintesi della melanina) [9].

Le mutazioni che diminuiscono l'attività della proteina *tumor suppressor*, retinoblastoma (RB), sono comuni sia nelle forme familiari che in quelle sporadiche. Il gene *CDKN2A* è mutato in circa il 40% degli alberi genealogici con trasmissione autosomica dominante del melanoma. *CDKN2A* è un locus molto complesso che codifica per tre differenti *tumor suppressor*, p15/INK4b, p16/INK4a e p14/ARF. La perdita di funzione di p16/INK4a, è

chiaramente collegata al melanoma, ed evidenze sperimentali supportano anche l'importanza della perdita di funzione di p14/ARF.

Un gruppo molto comune di lesioni molecolari identificate nei melanomi sporadici, è associato a un'attivazione costitutiva della via di segnale di RAS e di PI-3K/AKT, che regolano la crescita e la sopravvivenza cellulare. Le mutazioni attivanti il gene *BRAF*, che codifica per una serina/treonina chinasi a valle di RAS, sono state identificate nel 60-70% dei melanomi. Lesioni molecolari di entrambi i tipi, sono probabilmente un evento necessario ma non sufficiente all'insorgenza del melanoma. È chiaro quindi, che devono essere identificate ancora altre vie di segnale associate al processo di trasformazione neoplastica.

1.2 I farmaci anti-infiammatori non steroidei (FANS)

Studi recenti hanno dimostrato che l'uso frequente di farmaci antiinfiammatori è associato a un'incidenza minore del tumore del colon-retto e a una probabilità più bassa di recidiva [11-13]. I farmaci antinfiammatori si suddividono in due categorie in base alla loro struttura: non steroidei (FANS) e steroidei (cortisonici). I FANS (Figura 2) sono un gruppo eterogeneo di molecole, spesso chimicamente non correlate, ma accomunate dall'azione terapeutica e dagli effetti collaterali indotti dalla loro somministrazione [14]. Il meccanismo di azione di tali farmaci si basa sull'inibizione delle ciclossigenasi (COX). Esistono due forme di ciclossigenasi denominate COX-1 e COX-2. La COX-1 è espressa nella mucosa gastrica, bronchiale, nel parenchima renale e a livello ematico. Questa forma costitutiva è responsabile della produzione fisiologica delle prostaglandine coinvolte nella modulazione locale di funzioni quali la secrezione di acido e di muco nello stomaco, l'aggregazione piastrinica e la funzionalità renale. La COX-2 è la forma inducibile, normalmente non espressa nelle cellule differenziate, ma che è rapidamente indotta dagli

stimoli pro-infiammatori in diversi tipi cellulari come macrofagi, fibroblasti e cellule endoteliali.

L'azione antinfiammatoria dei FANS è associata all'inibizione della COX-2, mentre gli effetti collaterali, soprattutto quelli gastrici e renali, sembrano dipendere dall'inibizione della COX-1. Nell'ambito dei FANS esiste una diversa selettività verso la COX-1 e COX-2. Il più importante inibitore specifico della COX-1 è l'acido acetilsalicilico (aspirina). I FANS "tradizionali" (ibuprofene, diclofenac) sono inibitori non specifici delle COX. Inibitori preferenziali della COX-2 sono il nimesulide e il meloxicam. Infine, il rofecoxib e il celecoxib, farmaci di ultima generazione, sono inclusi tra gli inibitori selettivi della COX-2; essi hanno effetti antinfiammatori e analgesici, ma non sono gastrolesivi e sono privi di attività antiaggregante piastrinica.

Il diclofenac è un farmaco antinfiammatorio non steroideo (FANS) utilizzato ampiamente nella pratica clinica. Lavori recenti hanno evidenziato che i FANS possono influire negativamente sullo sviluppo di alcuni tumori [15,16] attraverso l'induzione dell'apoptosi e l'arresto del ciclo cellulare [17,18]. Il diclofenac, in particolare, esibisce un effetto citotossico su diverse linee cellulari attraverso un'alterazione dello stato redox cellulare [19,20]. Il nostro gruppo di ricerca ha inoltre evidenziato che il diclofenac induce apoptosi in una linea cellulare di neuroblastoma, SH-SY5Y, provocando una disfunzione mitocondriale [21]. Il trattamento delle SH-SY5Y con diclofenac aumenta i livelli intracellulari di specie reattive dell'ossigeno (ROS) e riduce sia i livelli proteici sia i livelli di attività enzimatica dell'enzima antiossidante mitocondriale superossido dismutasi 2 (SOD2).

1.3 Le specie reattive dell'ossigeno

Gli effetti dannosi dell'ossigeno sono rimasti oscuri fino alla pubblicazione della teoria dei radicali liberi di Gershman del 1954; questa afferma che la tossicità dell'ossigeno è dovuta ad alcune sue forme parzialmente ridotte [22]. Nello stesso periodo, mediante misure di risonanza paramagnetica elettronica (una tecnica spettroscopica in grado di rilevare specie chimiche con un elettrone spaiato), in diverse varietà di materiali biologici liofilizzati [23], sono stati rilevati segnali attribuibili alla presenza di radicali liberi. Un forte interesse nei confronti del ruolo svolto dalle specie reattive dell'ossigeno (ROS) nei sistemi biologici derivò anche dagli studi di Harman, i quali suggerirono che i radicali liberi erano coinvolti nel processo dell'invecchiamento [24]. La scoperta dell'enzima superossido dismutasi da parte di McCord e Fridovich nel 1969 fornì ulteriori evidenze riguardo all'importanza dei radicali liberi nei sistemi viventi [25]. Nel 1977, infine, Mittal e Murad fornirono la prova che il radicale idrossile ($HO^{\bullet}$) stimolava l'attivazione della guanilato ciclasi e la formazione del "secondo messaggero" guanosina monofosfato ciclico (cGMP) [26]. Le cellule, quindi, non solo si sono adattate alla presenza dei radicali liberi, ma hanno anche sviluppato diversi meccanismi per usufruire vantaggiosamente delle loro funzioni di secondi messaggeri, in diversi processi fisiologici.

I radicali liberi possono essere definiti come molecole o frammenti molecolari, contenenti uno o più elettroni spaiati negli orbitali atomici o molecolari [27]. Questi elettroni solitamente provocano un considerevole grado di reattività. I radicali derivati dall'ossigeno rappresentano la classe più importante di specie radicaliche generate nei sistemi viventi [28]. L'ossigeno molecolare possiede una configurazione elettronica unica, ed è una molecola termodinamicamente instabile. L'aggiunta di un elettrone alla forma biatomica genera il radicale superossido ($O_2^{\bullet -}$) [29]. L'anione superossido, i cui livelli aumentano sia durante i processi metabolici, che in seguito all'attivazione dell'ossigeno indotta

dall'irradiazione elettromagnetica, è considerato il ROS primario. $O_2^{\bullet-}$ è in grado di interagire ulteriormente con altre molecole, per generare ROS "secondari", sia direttamente che attraverso un processo catalizzato da enzimi o metalli [30].

Le specie reattive dell'ossigeno e dell'azoto, rispettivamente ROS (reactive oxygen species) e RNS (reactive nitrogen species), sono normalmente prodotti da enzimi strettamente regolati all'interno della cellula, come l'ossido nitrico sintasi (NOS) e la NAD(P)H ossidasi [31]. I ROS e gli RNS, a concentrazioni fisiologiche, sono coinvolti nella risposta al danno cellulare, nella difesa da agenti infettivi, nell'attivazione di numerosi *pathways* cellulari, e nell'induzione della risposta mitogenica. Al contrario, una sovrapproduzione di ROS e RNS provoca una condizione di stress ossidativo e stress nitrosativo, ed è responsabile di alterazioni strutturali e funzionali di lipidi, proteine e DNA. Un'alterazione dell'equilibrio redox cellulare, responsabile della condizione di stress, origina sia da un aumento nella produzione di ROS e RNS, che da una riduzione o da alterazione funzionale dei sistemi antiossidanti cellulari. Paradossalmente, varie attività mediate dai ROS proteggono la cellula dallo stress ossidativo indotto dai ROS stessi, e sono utili a ristabilire o mantenere il "equilibrio redox" (anche definito "omeostasi redox"). La doppia natura dei ROS è estensivamente documentata in letteratura: i ROS possono agire da secondi messaggeri inducendo e mantenendo il fenotipo oncogenico delle cellule cancerose, oppure possono indurre senescenza cellulare e apoptosi, fungendo quindi da specie anti-tumorigeniche.

L'anione superossido è prodotto principalmente dai fagociti, ad opera dell'enzima NADPH ossidasi, e all'interno dei mitocondri, a livello della catena di trasporto degli elettroni. Nel corso della fosforilazione ossidativa, infatti, una piccola quota di elettroni riduce parzialmente l'ossigeno portando alla formazione del radicale libero superossido, che è implicato nella patogenesi di numerose malattie. Il radicale superossido, infatti, è capace di

inattivare i nuclei ferro-zolfo delle proteine, provocando la produzione di ferro ridotto libero all'interno della cellula:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

generando il radicale idrossile ($HO^{\bullet}$) attraverso la reazione di Fenton:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-}$$

Il radicale idrossile, la forma neutra dello ione idrossido, possiede un'elevata reattività, e ciò lo rende un radicale molto pericoloso, con un'emivita *in vivo* di pochi secondi. Per questo motivo, quando è prodotto, reagisce soprattutto a livello del suo sito di formazione.
Altri radicali reattivi derivati dall'ossigeno sono quelli appartenenti al gruppo dei radicali perossili ($ROO^{\bullet}$). Il più semplice di questi è $HOO^{\bullet}$, la forma protonata del superossido ($O_2^{\bullet -}$) ed è generalmente denominato anche radicale idroperossile o radicale peridrossile. Questo radicale dà inizio alla perossidazione lipidica, con conseguente aumento di ROS e RNS.
I perossisomi sono notoriamente il sito di produzione di H_2O_2, e in condizioni fisiologiche partecipano a varie funzioni metaboliche, quali l'ossidazione degli acidi grassi a catena lunga durante la β-ossidazione, la sintesi di colesterolo e acidi biliari, la produzione di plasmalogeni. Il consumo di ossigeno nel perossisoma porta alla produzione di H_2O_2, che è poi trasformato in O_2 e H_2O dalla catalasi. Il mantenimento e il controllo di un fine equilibrio del numero e dell'attività degli enzimi dei perossisomi è necessario affinché la produzione dei ROS sia regolata. Quando i perossisomi sono danneggiati e il livello degli

enzimi preposti alla dismutazione del perossido d'idrogeno si riduce, l'H_2O_2 è rilasciata nel citosol, e contribuisce in modo rilevante allo stress ossidativo.

1.4 Sistemi antiossidanti

Alte concentrazioni di ROS possono indurre danni agli acidi nucleici, ai lipidi e alle proteine. Il radicale idrossile reagisce con il DNA, danneggiando le purine, le pirimidine e il deossiribosio. Le alterazioni permanenti indotte dai ROS al materiale genetico contribuiscono alla carcinogenesi e all'invecchiamento cellulare. Il danno però non coinvolge solo il DNA, i ROS, infatti, reagiscono anche con i grassi polinsaturi dei fosfolipidi di membrana, e possono quindi alterare la permeabilità della membrana agli ioni. I ROS e gli RNS, infine, modulano la funzionalità delle proteine reagendo con le catene laterali di aminoacidi, in particolare quelle contenenti residui di cisteina, provocando la formazione di ponti disolfuro intracatena e intercatena.

Poiché i radicali liberi sono prodotti continuamente nel corso del metabolismo cellulare, la cellula ha sviluppato dei sistemi di difesa antiossidante per proteggersi da ROS e RNS o correggere i danni causati da essi. I meccanismi antiossidanti cellulari sono sia di tipo enzimatico che non enzimatico. Tra i primi rientrano le superossido dismutasi (SOD), il sistema tioredossina/tioredossina reduttasi (trx/trxR), la glutatione perossidasi (GPx), la catalasi (CAT), mentre i secondi sono rappresentati dall' acido ascorbico (vitamina C), α-tocoferolo (vitamina E), glutatione (GSH), carotenoidi, flavonoidi, e altri antiossidanti. In condizioni normali si stabilisce un fine equilibrio tra produzione di ROS/RSN ed eliminazione ad opera dei sistemi antiossidanti; il controllo costante di tale equilibrio o "omeostasi redox" è fondamentale per la sopravvivenza degli organismi e per la loro salute.

Tra gli enzimi antiossidanti, le superossido dismutasi (SOD) rivestono un ruolo cruciale in quanto preposte all'eliminazione dell'anione superossido, il primo ROS che si forma dal metabolismo dell'ossigeno. Le SOD catalizzano la seguente reazione:

$$2\ O_2^{\bullet -} + 2\ H^+ \rightleftharpoons O_2 + H_2O_2$$

Esse, rappresentano uno tra i più importanti sistemi enzimatici antiossidanti cellulari. Le SOD sono dei metallo-enzimi, e presentano nel sito attivo diversi cofattori, quali: rame (Cu), zinco (Zn), manganese (Mn), ferro (Fe) o nichel (Ni).

Nell'uomo sono presenti tre forme di SOD, con localizzazioni cellulari differenti: la SOD1, presente nel citosol, la SOD2 presente nel mitocondrio, la SOD3 presente nello spazio extracellulare. La SOD1 e la SOD3 contengono all'interno del loro nucleo catalitico, gli ioni metallici Cu e Zn, mentre la SOD2 contiene Mn.

La manganese superossido dismutasi (Mn-SOD o SOD2), è un enzima tetramerico localizzato nella matrice mitocondriale. Essa è sintetizzata nel nucleo come precursore contenente all'estremità N-terminale un peptide segnale rappresentato da una sequenza di 24 residui amminoacidici. In seguito alla rimozione del peptide segnale, si genera la forma matura della proteina che poi si localizza nel mitocondrio. La SOD2 rappresenta uno tra i più importanti enzimi antiossidanti, perché l'intensa attività respiratoria che si svolge nei mitocondri degli organismi aerobi rende quest'organulo il principale sito di produzione dei ROS intracellulari. Il mitocondrio è un organulo fondamentale per la fisiologia cellulare in quanto regola la produzione di energia e l'apoptosi. Il controllo del livello dei ROS ad opera della SOD2, contribuisce a preservare l'integrità strutturale e funzionale del mitocondrio, rappresentando un enzima di fondamentale importanza nella fisiologia dell'intera cellula. In assenza della SOD2, i livelli del radicale superossido aumentano e la

cellula può subire una serie di modificazioni che ne compromettono la vitalità. Topi "*knock-out*" per la SOD2 muoiono durante lo sviluppo embrionale [32]. L'aumento dell'espressione della SOD2, inoltre, blocca l'apoptosi indotta da prolina ossidasi, dal TNF-α, dalla IL-3 o dalle radiazioni ionizzanti [33], e durante l'apoptosi mediata da FAS, la SOD2 è inattivata da una degradazione caspasi-specifica [34]. Comunque il potenziale ruolo della SOD2 nei meccanismi di sviluppo del cancro, è controverso [35]. I livelli della SOD2 sembrano diminuire in alcune linee cellulari cancerose, e l'induzione della sua espressione sembra essere in grado di sopprimere fenotipi maligni in alcuni modelli sperimentali; questo enzima è stato considerato, perciò, come un *tumor suppressor*. Inoltre, l'aumento di espressione della SOD2 è stato correlato a una risposta cellulare allo stress ossidativo tipico nelle cellule cancerose. L'incremento dell'attività della SOD2 causa una diminuzione del contenuto intracellulare di superossido, e riduce la stimolazione della crescita cellulare mediata dai ROS. È stato quindi ipotizzato che la SOD2 possa ridurre il tasso di proliferazione delle cellule cancerose indirettamente, attraverso la riduzione dei ROS, diversamente dai classici *tumor suppressor*, che regolano la crescita cellulare modulando l'espressione di alcuni geni nelle cellule cancerose.

1.5 Il metabolismo delle cellule tumorali: l'importanza del mitocondrio

La sopravvivenza delle cellule tumorali è associata alla loro maggiore capacità, rispetto alle cellule normali, di utilizzare una vasta gamma di substrati energetici alternativi al glucosio. La circolazione sanguigna, infatti, non è in grado di apportare nutrienti sufficienti a soddisfare le elevate esigenze metaboliche del tumore; le cellule neoplastiche presenti nelle zone più interne della massa tumorale, deprivate di glucosio, non sono più in grado di sintetizzare ATP attraverso la fosforilazione ossidativa [36]. Per questo motivo nelle cellule tumorali è attivato uno "switch" metabolico che permette l'utilizzo di fonti

energetiche alternative (Figura 3). Ne consegue che, nei tumori molto aggressivi, l'elevata richiesta energetica causa, nei pazienti, cachessia e un'improvvisa perdita di peso.

Uno dei cambiamenti metabolici più studiati nelle cellule tumorali è rappresentato dall'incremento della glicolisi anche in presenza di elevate concentrazioni di O_2 [37-43]. Nel 1956 Warburg propose che l'aumento della glicolisi nelle cellule tumorali, è indotto anche dalla compromissione irreversibile della funzionalità mitocondriale. Da quel momento, è stato universalmente accettato, senza una valida conferma sperimentale, il concetto secondo cui la richiesta di ATP nella cellula tumorale è soddisfatta, in massima parte o anche nella totalità, dalla glicolisi. In molte linee cellulari tumorali, indubbiamente si osserva una diminuzione della funzionalità mitocondriale, ma questo fenomeno non è stato dimostrato univocamente in tutte le cellule tumorali. A causa dell'eterogeneità genetica delle cellule tumorali, è quindi fondamentale un'analisi funzionale dell'efficienza della fosforilazione ossidativa per ciascun tipo di tumore, e valutare se l'aumento dell'attività glicolitica è effettivamente associato ad una riduzione della funzionalità mitocondriale. Nella maggior parte delle cellule tumorali, all'alto tasso di glicolisi [44-46] è associato un livello elevato di diversi enzimi glicolitici [43,47,48]. Per contro, pochi sono gli studi riguardanti l'aumento dei processi catabolici volti alla produzione alternativa di ATP nelle cellule tumorali, quali ad esempio l'ossidazione degli acidi grassi, responsabile spesso dei fenomeni di cachessia caratteristici della patologia neoplastica [49]. Poiché la glicolisi costituisce la fonte principale per la sintesi di ATP nelle cellule cancerose, il glucosio costituisce la fonte di carbonio principale utilizzata dal tumore. Oltre al glucosio trasportato dal sangue e metabolizzato molto velocemente, sono utilizzate altre fonti di carbonio, quali il lattato, gli aminoacidi, gli acidi grassi, i corpi chetonici [40,50,51]. Le vie ossidative mitocondriali e la fosforilazione ossidativa rivestono comunque un ruolo molto importante nella fisiologia della cellula tumorale, anche se la glicolisi rappresenta la via

metabolica principale per soddisfare l'elevata richiesta di ATP. Nella cellula tumorale l'alterazione del metabolismo porta a un incremento dei livelli intracellulari di ROS rispetto alle cellule normali [52]. I ROS, non solo regolano la proliferazione cellulare e contribuiscono al processo di trasformazione neoplastica [53], ma possono anche attivare i processi di senescenza, di autofagia e di morte cellulare, necessari per eliminare le cellule danneggiate e neoplastiche [54]. Negli ultimi anni molti studi sono stati rivolti all'individuazione di farmaci, specifici per il mitocondrio, capaci di alterare ulteriormente il metabolismo energetico e/o ossidativo delle cellule neoplastiche. I mitocondri sono, infatti, la fonte principale di ROS nella cellula. I livelli maggiori di ROS misurati nelle cellule neoplastiche, sono, in gran parte, una diretta conseguenza dell'elevato tasso metabolico e della disfunzione mitocondriale che caratterizza la massa tumorale. Farmaci che inducono, quindi, un ulteriore incremento di ROS, e/o molecole capaci di regolare negativamente i meccanismi anti-ossidanti, potrebbero indurre selettivamente nelle cellule neoplastiche, la morte cellulare. Recentemente, sono stati identificati molti composti che hanno come target il mitocondrio delle cellule tumorali; tali molecole sono state denominate "mitocans" (Figura 4). La caratterizzazione delle differenze metaboliche che influenzano la funzionalità mitocondriale tipica delle cellule neoplastiche, può quindi contribuire allo sviluppo di farmaci con una maggiore selettività verso le cellule tumorali.

1.6 Processi programmati di morte cellulare: l'apoptosi

L'apoptosi, ovvero la morte cellulare programmata, è un processo che regola tutta la vita di un organismo, dallo sviluppo embrionale. Essa è essenziale per garantire il corretto differenziamento cellulare nei diversi tessuti e per mantenere l'omeostasi cellulare degli organismi pluricellulari. Quando un danno altera irreversibilmente la funzionalità di una cellula, questa attiva l'apoptosi affinché il danno non si trasmetta alla progenie. Le vie di

segnale che attivano l'apoptosi sono geneticamente conservate e le loro alterazioni sono coinvolte in un gran numero di patologie, quali ad esempio le neoplasie e le malattie neurodegenerative. La cellula apoptotica perde rapidamente volume condensandosi, si stacca dalle cellule vicine ed espone molecole normalmente nascoste o poco espresse sulla membrana plasmatica, come ad esempio la fosfatidilserina. Nel nucleo della cellula apoptotica, la cromatina si condensa e il DNA è frammentato in porzioni di 180-200 bp, lunghezza corrispondente ai tratti di DNA internucleosomale. Nell'ultima fase del processo apoptotico si formano delle vescicole citoplasmatiche chiamate corpi apoptotici, che possiedono un contenuto ipodiploide di DNA. I corpi apoptotici sono riconosciuti e rimossi dalle cellule fagocitiche, evitando in questo modo possibili risposte infiammatorie e processi flogistici secondari, come avviene invece durante la necrosi.

Il processo apoptotico è generalmente suddiviso nelle seguenti fasi:

- Induzione: numerosi fattori, sia di origine endogena che esogena, svolgono un ruolo chiave nell'attivazione dell'apoptosi (p53, TNF, farmaci, radiazioni UV, shock da calore, ipossia, riduzione dei fattori di crescita cellulare)
- Esecuzione: la fase di esecuzione è caratterizzata da una serie di reazioni enzimatiche a cascata che, una volta innescate causano la morte cellulare
- Fagocitosi dei corpi apoptotici mediata dalle cellule fagocitiche

Nella fase dell'esecuzione intervengono le caspasi (proteasi cisteina-dipendenti e aspartato specifiche), la cui attività catalitica dipende dalla presenza nel sito attivo dell'enzima di un residuo di cisteina altamente conservato (QACRG), indispensabile per la catalisi. Il taglio proteolitico sulla proteina bersaglio avviene a livello di uno specifico residuo di acido aspartico. Le caspasi (nell'uomo sono stati identificati 14 membri, di cui solo 7 coinvolti nell'apoptosi) sono presenti sotto forma di zimogeni o pro-caspasi (precursori inattivi). In seguito a tagli proteolitici dei precursori inattivi si ottengono le caspasi attive. Le caspasi

effettivamente coinvolte nel suicidio cellulare sono distinte in caspasi iniziatrici (caspasi 2, 8, 9, 10), ed effettrici (caspasi 3, 6, 7). In alcuni casi il processo apoptotico è attivato attraverso un meccanismo caspasi-indipendente. In questo processo intervengono proteine pro-apoptotiche quali AIF (apoptosis inducing factor), CypA ed EndoG, che si associano nel nucleo inducendo direttamente la condensazione della cromatina, la degradazione del DNA e, quindi, il processo apoptotico [55].

Anche se i fattori di attivazione dei processi apoptotici possono essere molteplici, le vie di attivazione della morte programmata sono di due tipi:

- la via estrinseca, attivata dalle interazioni di ligandi extracellulari con recettori specifici (recettori di morte) esposti sulla membrana plasmatica
- la via intrinseca, che prevede il coinvolgimento del mitocondrio

Nella prima via la caspasi 8 agisce come iniziatrice della fase di esecuzione del processo apoptotico, mentre in quella intrinseca tale ruolo è svolto dalla caspasi 9.

La via di morte cellulare regolata dal mitocondrio, può essere attivata da una serie di fattori esterni non legati alla stimolazione di specifici recettori cellulari, come ad esempio radiazioni, radicali liberi, infezioni virali, carenza di fattori di crescita/siero, farmaci. Questi stimoli provocano delle variazioni nel potenziale della membrana mitocondriale interna, attraverso l'apertura di un poro di transizione della permeabilità mitocondriale (MPT); questo evento porta alla perdita del potenziale transmembrana mitocondriale ($\Delta\Psi_m$), al rilascio dal mitocondrio di proteine pro-apoptotiche e all'interruzione del processo di produzione di energia. Le proteine che sono rilasciate dal mitocondrio possono essere raggruppate in due categorie. La prima comprende proteine in grado di attivare l'apoptosi caspasi-dipendente, e include il citocromo *c* (cyt *c*) e Smac/DIABLO (second mitochondria-derived activator of caspases). Durante l'attivazione di questa via, in seguito alla formazione del poro mitocondriale, il cyt *c* fuoriesce dal mitocondrio e nel citosol si

aggrega con Apaf-1 e con la procaspasi 9, formando un complesso macromolecolare detto apoptosoma, capace di iniziare la cascata di attivazione delle caspasi, a partire dalla caspasi 9. La seconda comprende altre proteine pro-apoptotiche che agiscono in modo caspasi-indipendente, come AIF (apoptosis inducing factor) ed EndoG (endonuclease G), che intervengono in una fase tardiva dell'apoptosi quando le cellule sono già indirizzate alla morte [55].

Scopo della ricerca

In una cellula neoplastica le esigenze energetiche sono maggiori rispetto a quelle di una cellula normale. La cellula cancerosa assolve tali necessità energetiche sia attraverso la glicolisi aerobica sia mediante l'attivazione di vie cataboliche alternative. Quando il livello di ATP è inferiore a una certa soglia, la cellula normale riesce a sopravvivere mentre la cellula tumorale muore. Ciò suggerisce che le cellule cancerose, possedendo una soglia energetica maggiore rispetto a quella delle cellule normali, siano più sensibili agli agenti che riducono i livelli di ATP. Lo studio delle basi molecolari che regolano lo "switch" metabolico caratteristico delle cellule neoplastiche può quindi contribuire all'identificazione di target terapeutici specifici per la cellula tumorale [36]. I mitocondri, oltre ad essere le centrali energetiche della cellula, modulano i processi di senescenza, autofagia e apoptosi. Poiché è stato osservato che le alterazioni metaboliche della cellula neoplastica influenzano la funzionalità mitocondriale, molti studi sono stati rivolti all'identificazione di nuove molecole il cui target è il mitocondrio. Alcuni composti, detti "mitocans" (Figura 4) alterano la funzionalità mitocondriale e inducono il processo apoptotico, preferenzialmente nelle cellule tumorali [56].

Negli ultimi anni, è stato riportato che i FANS possono regolare negativamente lo sviluppo del tumore [15,16], inducendo apoptosi e arresto del ciclo cellulare [17,18]. Il diclofenac, uno dei FANS più usati come analgesico, esercita un effetto citotossico e anti-proliferativo in molte linee tumorali, alterando lo stato redox cellulare e la funzionalità mitocondriale [19,20].

Scopo dell'attività di ricerca, durante il mio periodo di dottorato, è stato quello di analizzare gli effetti citotossici del diclofenac, in linee cellulari di melanoma umano A2058 e SAN e in una linea cellulare di fibroblasti umani immortalizzati non tumorali, BJ-5ta. In particolare, sono stati studiati gli eventi che inducono l'apoptosi ed è stato valutato il coinvolgimento del mitocondrio in tale processo, con particolare attenzione agli eventuali effetti sui livelli e sull'attività della SOD2.

Materiali e metodi

2.1 Prodotti chimici e reagenti

I terreni di coltura Dulbecco's Modified Eagle's Medium (DMEM) e Roswell Park Memorial Institute 1640 (RPMI-1640), il siero fetale bovino (FBS), la L-glutammina, la penicillina G, la streptomicina e la tripsina, sono stati acquistati dalla ditta Lonza. L'igromicina B è stata acquistata dalla ditta Invitrogen. Il diclofenac è stato acquistato dalla ditta Calbiochem. Il terreno di coltura Medium 199 (M199), lo ioduro di propidio (PI), la diclorofluoresceina diacetato (DCFH-DA) e il diidroetidio (DHE), sono stati acquistati dalla ditta Sigma Aldrich. Il cocktail d'inibitori di proteasi è stato acquistato dalla ditta Roche diagnostics. I kit fluorimetrici per il dosaggio della caspasi-3 e caspasi-9, sono stati acquistati dalla ditta BioVision. L'anticorpo policlonale anti SOD2 prodotto in rabbit è stato acquistato dalla ditta Millipore; l'anticorpo monoclonale anti GAPDH prodotto in rabbit, è stato acquistato dalla ditta Cell Signaling; l'anticorpo monoclonale anti Bcl-2 prodotto in mouse, l'anticorpo policlonale anti β-actina prodotto in goat, l'anticorpo policlonale anti citocromo *c* ossidasi IV (COX-IV) prodotto in goat, l'anticorpo monoclonale anti citocromo *c* prodotto in mouse e tutti gli anticorpi secondari coniugati con perossidasi di rafano, sono stati acquistati dalla ditta Santa Cruz biotechnology. Tutti gli altri reagenti chimici di grado analitico sono stati acquistati dalla ditta Sigma Aldrich.

2.2 Colture cellulari

Le cellule di melanoma umano A2058, cortesemente fornite dal CEINGE di Napoli, e le cellule SAN [57], derivano da una metastasi linfonodale. Le cellule A2058 e SAN sono state coltivate, rispettivamente, in DMEM e RPMI-1640, integrato con FBS 10%, 2mM L-

glutammina, 100 IU/mL di penicillina G e 100 µg/mL di streptomicina, in un incubatore con atmosfera umidificata a 37 °C e 5% di CO_2. Le cellule BJ-5ta [58], una linea cellulare di fibroblasti di cute umana, immortalizzata con hTERT, sono state coltivate in un terreno di coltura composto da una miscela di DMEM e M199 in rapporto 4:1, integrato con FBS 10%, 4 mM L-glutammina 4,5 g/L di glucosio, 1,5 g/L di bicarbonato di sodio, 100 UI/mL di penicillina G, 100 µg/mL di streptomicina e 0,01 mg/mL di igromicina B, in un incubatore con atmosfera umidificata a 37 °C e 5% di CO_2. Tutte le cellule sono state splittate e seminate in piastre da 75 cm^2 ogni 3 giorni e usate durante la loro fase di crescita esponenziale. Tutti i trattamenti alle cellule sono stati eseguiti 24 ore dopo la loro semina.

2.3 Valutazione dell'apoptosi

Per la determinazione del numero di nuclei apoptotici, le cellule sono state utilizzate in piastre multi-pozzetto da 96 alla concentrazione di $1x10^4$ per ogni pozzetto; al termine di ciascun trattamento, le cellule sono state staccate per tripsinizzazione, la sospensione cellulare è stata centrifugata e lavata con fosfato di sodio 10 mM, pH 7.2 contenente 150 mM NaCl (PBS), ed il pellet cellulare così ricavato è stato risospeso in una soluzione ipotonica lisante contenente PI 50 µg/mL. Dopo un'incubazione a 4 °C per 30', le cellule sono state analizzate tramite un citofluorimetro per valutare la presenza di nuclei con contenuto di DNA ipodiploide [59].

2.4 Misurazione del contenuto intracellulare di ROS

I livelli intracellulari di ROS sono stati misurati utilizzando le sonde fluorescenti sensibili all'ossidazione, DHE e DCFH-DA [60,61].

In breve, le cellule sono state seminate in piastre multi-pozzetto da 6 alla concentrazione di $3x10^5$ cellule per pozzetto ed incubate con 150 µM di diclofenac, per tempi differenti.

DHE è stato aggiunta al buio alla concentrazione finale 10 μM, 30 minuti prima dell'aggiunta del farmaco. DCFH-DA è stata aggiunta al buio alla concentrazione finale 10 μM 30 minuti prima del termine dell'incubazione con il farmaco. Le cellule, in seguito, sono state raccolte, lavate in PBS, e infine risospese in 500 μL di PBS per la successiva analisi fluorimetrica. La misurazione del contenuto di ROS è stata eseguita con uno spettrofotometro a fluorescenza Cary Eclipse (Varian). Le lunghezze d'onda di eccitazione ed emissione utilizzate per DHE sono, rispettivamente, 392 nm e 410 nm, mentre per DCFH-DA sono rispettivamente, 485 nm e 538 nm; in entrambi i casi, le slits di eccitazione e di emissione sono state impostate a 10 nm.

2.5 Estratti cellulari totali e analisi mediante Western blotting

Le cellule sono state seminate in piastre multi-pozzetto da 6 alla concentrazione di $3x10^5$ cellule per pozzetto. Al termine di ciascun trattamento le cellule sono state raccolte, lavate con PBS e poi lisate con RIPA buffer modificato (Tris-HCl 50 mM, pH 7.4, NaCl 150 mM, Nonidet P-40 1%, Na deossicolato 0,25%, Na_3VO_4 1 mM e NaF 1 mM), integrato con inibitori di proteasi e incubato per 30 minuti in ghiaccio. I surnatanti ottenuti dopo centrifugazione a 12000 g per 30 minuti a 4 °C, costituiscono l'estratto proteico cellulare totale. La concentrazione proteica è stata determinata attraverso il metodo di Bradford [62], utilizzando albumina di siero bovino (BSA) come standard di riferimento. Le analisi mediante Western blotting sono state eseguite utilizzando uguali quantità di estratto proteico. In breve, i campioni proteici sono stati risospesi in loading buffer riducente contenente SDS, sottoposti a SDS-PAGE al 14% e poi trasferiti su una membrana Immobilon P (Millipore). Le membrane sono state incubate con gli anticorpi specifici a 4 °C o.n. e, in seguito, con gli specifici anticorpi secondari coniungati con perossidasi di rafano, a temperatura ambiente, per 1 ora. Le membrane sono state quindi analizzate

attraverso una reazione di chemoluminescenza intensificata usando il kit Immobilon Western Chemiluminescent HRP Substrate (Millipore); i segnali sono stati visualizzati attraverso auto-radiografia.

2.6 Dosaggio dell'attività delle caspasi-3 e -9

L'attività enzimatica della caspasi-3 e -9, durante il trattamento con diclofenac, è stata misurata utilizzando kit per il dosaggio fluorimetrico, seguendo le istruzioni fornite dal produttore. In breve, sono state seminate $2x10^6$ cellule in piastre da 75 cm^2 e incubate con 150 μM di diclofenac. Al termine di ciascun'incubazione le cellule sono state raccolte, lavate in PBS, e lisate a 4 °C in un buffer di lisi cellulare fornito dal produttore, integrato con inibitori di proteasi. I lisati cellulari (150 μg) sono stati incubati per 2 ore a 37 °C, al buio, con 50 μM di DEVD-AFC o 50 μM di LEHD-AFC, che sono i substrati fluorescenti, rispettivamente, della caspasi-3 e caspasi-9. Le misure sono state realizzate con uno spettrofotometro a fluorescenza Cary Eclipse (Varian). Le lunghezze d'onda utilizzate sono 400 nm per l'eccitazione e 505 nm per l'emissione; le slits di eccitazione e di emissione sono state impostate a 10 nm.

2.7 Small interference RNA

Gli small-interfering RNA specifici per la SOD2 (SOD2 siRNA), e i siRNA aspecifici di controllo (NS siRNA), sono stati acquistati dalla ditta Dharmacon. La trasfezione cellulare con siRNA è stata eseguita utilizzando Metafectene SI reagent, seguendo le istruzioni fornite dal produttore. In breve, le cellule sono state seminate in piastre multi-pozzetto da 12 alla concentrazione di $6x10^4$ cellule per pozzetto, in mezzo completo o.n. Il giorno seguente, 4 μL di Metafectene SI reagent e 70 nM di siRNA, sono stati aggiunti a 60 μL di SI buffer, e incubati a temperatura ambiente per 15 minuti, quindi in seguito la miscela di

reazione è stata aggiunta alle cellule. Dopo 72 ore, il mezzo di coltura è stato sostituito con del nuovo mezzo completo e le cellule sono state incubate in presenza o assenza di 150 μM di diclofenac per ulteriori 24 ore. Al termine del trattamento farmacologico, le cellule sono state raccolte e divise in due aliquote: una, utilizzata per verificare tramite Western blotting il grado di silenziamento della SOD2, l'altra per misurare l'apoptosi tramite incorporazione di PI.

2.8 Frazionamento subcellulare e analisi mediante Western blotting

Sono state seminate $2x10^6$ cellule in piastre da 75 cm^2. Dopo il trattamento, le cellule sono state raccolte, lavate in PBS e risospese in buffer M (Hepes 5 mM pH 7.4, mannitolo 250 mM, EGTA 0,5 mM, BSA 0,1%), integrato con inibitori di proteasi e omogenizzato. L'omogenato è stato quindi centrifugato a 800 g per 10 minuti a 4 °C. Il surnatante così ricavato è stato poi ulteriormente centrifugato a 12000 g per 30 minuti a 4 °C. Il sopranatante ottenuto in questo modo, rappresenta la frazione citosolica, mentre il pellet, che costituisce la frazione mitocondriale, è stato lisato utilizzando RIPA buffer. La concentrazione proteica è stata determinata come indicato in precedenza. Aliquote di entrambe le frazioni (citosoliche e mitocondriali) sono state utilizzate per analisi mediante Western blotting.

2.9 Misurazione dell'attività enzimatica della SOD2

$2x10^6$ cellule sono state piastrate in piastre da 75 cm^2. Al termine del trattamento, le cellule sono state raccolte e centrifugate a 300 g a 4 °C per 5 minuti, lavate con PBS e la frazione mitocondriale è stata preparata come precedentemente descritto, con la seguente modifica: il pellet mitocondriale, risospeso in un buffer contenente fosfato di potassio 50 mM, pH 7.8 e EDTA 1 mM, è stato sonicato, centrifugato a 20000 g a 4 °C per 30 minuti, e il

surnatante così ottenuto, è stato utilizzato per il dosaggio dell'attività enzimatica SOD2. L'attività della SOD2 è stata misurata a 25 °C in un buffer contenente fosfato di potassio 100 mM, pH 7.8 e Na_2EDTA 0,1 mM, valutando l'inibizione della riduzione del citocromo *c* provocata dagli anioni superossido, generati con il metodo della xantina/xantina ossidasi [63,64]. 1 unità di attività della SOD2 rappresenta la quantità di enzima che provoca il 50% d'inibizione della riduzione del citocromo *c*.

2.10 Immunofluorescenza

$3x10^5$ cellule sono state seminate in una piastra multi-pozzetto da 6 nella quale sono stati posti vetrini da 22 mm di diametro. Al termine del trattamento farmacologico, le cellule sono state incubate con 80 nM di MitoTracker Red® (Invitrogen), a 37 °C per 1 ora, e poi lavate per tre volte con PBS. Le cellule sono state quindi fissate con paraformaldeide 4%, permeabilizzate con 0,1% di triton X-100 e incubate con donkey serum (Millipore) al 10 % per 1 ora a temperatura ambiente. I vetrini sono stati quindi incubati per 1 ora a 37 °C con un anticorpo primario policlonale prodotto in rabbit e diretto contro la SOD2, quindi per 1 ora a 37 °C con un anticorpo secondario coniugato con fluoresceina isotiocianato (FITC, Jackson Immunoresearch). Il montaggio del vetrino è stato eseguito utilizzando Vectashield®. Le cellule sono state quindi osservate utilizzando un microscopio confocale Zeiss LSM 510 con un obiettivo planopo 63X. Il laser è stato impostato a 488 nm per l'anticorpo coniugato con FITC ed a 543 nm per MitoTracker Red. Le immagini sono state acquisite contemporaneamente nei canali del verde e del rosso, e processate attraverso LSM software.

2.11 Analisi statistica

I dati numerici sono stati analizzati utilizzando il test *t* di Student e one-way ANOVA con correzioni di Bonferroni, per le comparazioni multiple.

Risultati

3.1 Analisi morfologica delle cellule A2058, SAN e BJ-5ta, trattate con diclofenac

Le cellule A2058, SAN e BJ-5ta sono state incubate per 24 e 48 ore con concentrazioni di diclofenac, comprese tra 50 μM e 200 μM. Successivamente, utilizzando un microscopio a contrasto di fase, sono stati osservati eventuali cambiamenti della morfologia cellulare, indotti dal farmaco. Dalla Figura 5 si evince che, dopo 48 ore, il trattamento con 150 μM di diclofenac provoca, nelle cellule di melanoma, degli evidenti cambiamenti morfologici tipici del processo apoptotico [65]. Inoltre, gli stessi cambiamenti morfologici compaiono già dopo 24 ore di trattamento con la stessa concentrazione di farmaco, o a concentrazioni di diclofenac inferiori (100 μM) dopo 48 ore di incubazione. Invece, tali modifiche morfologiche sono molto meno evidenti nelle cellule BJ-5ta trattate con il farmaco (Fig. 5). Questi dati suggeriscono che le cellule di melanoma sono più sensibili al potenziale pro-apoptotico del diclofenac, rispetto alla linea cellulare di fibroblasti non maligni.

3.2 Analisi dell'effetto citotossico del diclofenac sulle linee cellulari di melanoma

Per valutare la natura dei cambiamenti morfologici indotti dal diclofenac nelle linee cellulari di melanoma, le cellule trattate e non sono state incubate con ioduro di propidio (PI), e analizzate mediante citofluorimetria. L'analisi citofluorimetrica delle cellule A2058, SAN e BJ-5ta, incubate per 24 ore con concentrazioni crescenti di diclofenac, ha mostrato un incremento dose-dipendente del numero di nuclei apoptotici nelle cellule A2058 (Fig. 6a) e SAN (Fig. 6b), mentre nelle cellule BJ-5ta non si osserva induzione di apoptosi, persino in presenza della concentrazione di farmaco 200 μM (Fig. 6c). Per valutare la

cinetica d'induzione del processo apoptotico, le cellule sono state incubate con 150 μM di diclofenac (dose minima di farmaco capace di indurre l'apoptosi nelle cellule di melanoma), per tempi diversi. Dopo 24 ore di trattamento, il numero di nuclei con contenuto di DNA sub-diploide aumenta sia nelle cellule A2058 (Fig. 6d), sia nelle cellule SAN (Fig. 6e), mentre tale aumento non è stato osservato nelle cellule BJ-5ta (Fig. 6f). Inoltre, dopo 48 ore di trattamento, il processo apoptotico risulta ancora più evidente; nelle cellule A2058 la percentuale di apoptosi raggiunge il 39%, con un incremento di circa 8 volte rispetto alle cellule non trattate (Fig. 6d), mentre nelle stesse condizioni sperimentali, le cellule SAN esibiscono una maggiore sensibilità al trattamento farmacologico, mostrando il 71% di apoptosi (Fig. 6e). Per quanto riguarda le cellule BJ-5ta, il trattamento con diclofenac per 48 ore, induce soltanto il 18% di apoptosi, un incremento di appena 3 volte rispetto alle cellule non trattate con il farmaco (Fig. 6f).

L'effetto pro-apoptotico del diclofenac è stato valutato anche attraverso il dosaggio dell'attività enzimatica della caspasi-3, l'enzima più importante tra quelli coinvolti nell'esecuzione del processo apoptotico. Quando la caspasi-3 viene attivata, infatti, il programma di morte cellulare diviene irreversibile [66]. L'attività della caspasi-3 è stata misurata nelle cellule A2058, SAN e BJ-5ta dopo 24 ore di trattamento con 150 μM di diclofenac. I dati mostrati in Figura 7 indicano che il diclofenac induce un incremento dell'attività della caspasi 3 in entrambe le linee cellulari di melanoma A2058 e SAN, mentre nelle cellule BJ-5ta l'attività enzimatica non varia. E' interessante notare che anche l'incremento dell'attività enzimatica, indotto dal trattamento farmacologico, è risultato maggiore nelle SAN rispetto alle cellule A2058.

Per approfondire ulteriormente lo studio dell'effetto pro-apoptotico esercitato dal diclofenac, sono stati quindi analizzati i livelli della proteina anti-apoptotica Bcl-2 e della proteina pro-apoptotica Bax [65,66], e in particolare è stato valutato il rapporto tra Bcl-2 e

Bax, che regola l'attivazione del processo apoptotico [67]. Nelle cellule A2058 e SAN incubate con 150 μM di diclofenac per 24 ore, è stata riscontrata una riduzione dei livelli proteici di Bcl-2; in particolare, nelle cellule SAN tale evento è evidente anche dopo 48 ore di trattamento (Fig. 8a, b). Al contrario nelle cellule non maligne BJ-5ta i livelli di Bcl-2 restano invariati (Fig. 8a, b). I livelli di Bax, invece, aumentano nelle cellule A2058 dopo 24 e 48 ore di trattamento con il diclofenac, mentre restano pressoché invariati nelle cellule SAN; per quanto riguarda, le cellule BJ-5ta, i livelli di Bax diminuiscono dopo 24 ore, per aumentare dopo 48 ore di incubazione con il farmaco (Fig. 8a, c). Una valutazione del rapporto Bcl-2/Bax, permette di chiarire meglio il contributo esercitato da queste due proteine nell'attuazione del processo apoptotico. È stata eseguita, quindi, un'analisi del rapporto tra Bcl-2/Bax e, come mostrato in Figura 8d, è evidente una riduzione del rapporto dopo 24 ore di trattamento con il farmaco, sia nelle cellule A2058 che nelle cellule SAN, mentre nei fibroblasti non maligni BJ-5ta si osserva un lieve, ma significativo, aumento di tale rapporto. Dopo 48 ore di trattamento con il diclofenac, in tutte e tre le linee cellulari non si evidenzia nessuna variazione del rapporto Bcl-2/Bax.

Questi dati, complessivamente, confermano che il diclofenac esercita il suo effetto citotossico attraverso l'attivazione di un processo apoptotico, che coinvolge principalmente le cellule di melanoma.

3.3 Analisi dei livelli intracellulari di ROS durante il trattamento con diclofenac

Poiché è noto che i FANS alterano l'equilibrio redox di diverse linee cellulari [20,21,61], per chiarire i meccanismi attraverso i quali il diclofenac esercita l'effetto apoptotico, sono stati misurati i livelli intracellulari di ROS mediante l'uso delle sonde fluorescenti DHE e DCFH-DA. Le due sonde rivelano, rispettivamente, la presenza dell'anione superossido, del perossido d'idrogeno e dei ROS che derivano dall'anione superossido (Fig. 9).

L'analisi a tempi diversi con DHE delle cellule A2058 (Fig. 9a), SAN (Fig. 9b) e BJ-5ta (Fig. 9c) incubate con diclofenac, mostra un incremento precoce dell'intensità di fluorescenza soltanto nelle linee cellulari di melanoma. Nei fibroblasti BJ-5ta il farmaco non provoca alcuna variazione significativa dei livelli di anione superossido; al contrario, dopo 15 minuti dal trattamento farmacologico, si può osservare una riduzione di questo ROS. L'incremento del livello di ROS, nelle cellule di melanoma trattate con il farmaco, è stato rilevato anche mediante l'utilizzo della sonda DCFH-DA. Tale incremento è stato però osservato dopo tempi più lunghi d'incubazione con il diclofenac (Fig. 9d, e). Invece, il trattamento farmacologico, anche per tempi più lunghi, delle cellule BJ-5ta non induce alcun aumento del livello dei ROS intracellulari (Fig. 9f).

3.4 Effetti del diclofenac sui livelli dell'enzima SOD2

L'enzima mitocondriale SOD2 rappresenta uno tra i principali agenti antiossidanti [70]. Il nostro gruppo di ricerca, ha già dimostrato che il diclofenac provoca una diminuzione dei livelli della proteina SOD2 in una linea cellulare tumorale di neuroblastoma [21]. Gli effetti del diclofenac sulla SOD2 sono stati quindi valutati anche nei nostri sistemi cellulari. In Figura 10 è mostrata l'analisi attraverso Western blotting dei livelli proteici della SOD2 nelle cellule di melanoma e nei fibroblasti non maligni dopo trattamento con 150 μM di diclofenac per 24 e 48 ore. In entrambe le linee di melanoma c'è una significativa riduzione dei livelli proteici della SOD2 dopo 48 ore di trattamento. Il diclofenac non provoca, invece, cambiamenti significativi dei livelli della SOD2 nelle cellule BJ-5ta. Inoltre il trattamento con il farmaco non induce variazioni significative dei livelli della Cu/Zn SOD (SOD1) (dati non mostrati).

È stato quindi valutato se il diclofenac inducesse, oltre alla riduzione dei livelli proteici, anche una riduzione dell'attività enzimatica della SOD2. A questo scopo sono stati

preparati estratti proteici mitocondriali dalle cellule A2058, SAN e BJ-5ta incubate con 150 μM di diclofenac per 24 e 48 ore. I dati in Figura 11 dimostrano che dopo 48 di trattamento farmacologico c'è una riduzione significativa dell'attività enzimatica della SOD2 in entrambe le linee cellulari di melanoma, mentre non è stata riscontrata alcuna variazione di attività enzimatica negli estratti proteici delle cellule BJ-5ta. Inoltre, dopo 24 ore di trattamento con il diclofenac è stata rilevata una lieve riduzione dell'attività enzimatica della SOD2 soltanto negli estratti proteici mitocondriali delle cellule A2058 (dati non mostrati).

Per capire se la riduzione dei livelli di SOD2 contribuisca all'effetto pro-apoptotico del diclofenac, le cellule di melanoma A2058 e SAN, sono state trasfettate con siRNA specifici per il messaggero della SOD2. I dati mostrati in Figura 12, evidenziano che il solo silenziamento della SOD2, non induce il processo apoptotico, mentre se le cellule trasfettate con siRNA specifici per la SOD2 sono anche trattate con il diclofenac l'effetto pro-apoptotico del farmaco è notevolmente amplificato in entrambe le linee cellulari di melanoma. Questi risultati suggeriscono che in assenza di farmaco il solo silenziamento della SOD2 non ha effetti sulla vitalità cellulare, mentre in assenza della SOD2 l'effetto pro-apoptotico del diclofenac è amplificato.

3.5 Effetto del diclofenac sulla distribuzione subcellulare degli enzimi SOD2 e citocromo c, e sull'attività enzimatica della caspasi-9

Per analizzare i meccanismi molecolari che regolano il processo apoptotico indotto dal diclofenac, e, per valutare il coinvolgimento mitocondrio in questo processo, è stata studiata la localizzazione subcellulare, nelle cellule A2058 trattate con il farmaco, delle proteine mitocondriali citocromo *c e* SOD2 (Fig. 13). IL citocromo *c* è normalmente localizzato nel mitocondrio, ma in risposta ad alcuni stimoli pro-apoptotici, trasloca nel

citosol e induce l'attivazione della caspasi 9 [71,72]. La SOD2 è un tipico marcatore mitocondriale [73,74], e la sua traslocazione dal mitocondrio al citosol è poco documentata in letteratura. L'analisi attraverso Western blotting, degli estratti proteici citosolici e mitocondriali delle cellule A2058 trattate con 150 μM di diclofenac per 24 ore, dimostra che il trattamento farmacologico induce non solo la traslocazione citosolica del citocromo *c* ma sorprendentemente anche la traslocazione citosolica della SOD2 (Fig. 13a). Nelle cellule A2058 trattate con il farmaco, la comparsa della SOD2 nella frazione citosolica è associata a una riduzione dei livelli della proteina nella frazione mitocondriale (Fig. 13a). Risultati simili sono stati ottenuti con le cellule SAN, mentre nelle cellule BJ-5ta non è stata rilevata nessuna traslocazione del citocromo *c* o della SOD2 (dati non mostrati).

Per confermare ulteriormente la traslocazione citosolica della SOD2, sono stati effettuati esperimenti di immunofluorescenza. Le immagini mostrate in figura 14, confermano che il trattamento con il diclofenac induce la localizzazione citosolica della SOD2. L'analisi attraverso microscopia confocale, mostra che nelle cellule A2058, SAN e BJ-5ta non trattate con il farmaco, la proteina SOD2 è presente esclusivamente nel compartimento mitocondriale, mentre solo nelle cellule di melanoma, stimolate con il farmaco, è possibile evidenziare immunocolorazione specifica per la SOD2 nel citosol.

L'attivazione della caspasi-9 è un tipico evento della via intrinseca dell'apoptosi [75]. Nel nostro sistema sperimentale, è stata misurata l'attività enzimatica della caspasi-9 dopo trattamento con diclofenac. I dati presentati in Figura 15, indicano che l'attività della caspasi-9 aumenta in modo significativo in entrambe le linee cellulari di melanoma incubate per 24 ore, con 150 μM di diclofenac. Al contrario, nelle stesse condizioni sperimentali, nelle cellule non maligne BJ-5ta, non sono stati rilevati incrementi nell'attività della caspasi-9. Questi ultimi dati confermano inoltre che le due linee cellulari di melanoma hanno una diversa sensibilità al trattamento farmacologico, e suggeriscono

che il diclofenac influenza la funzionalità mitocondriale delle cellule di melanoma, inducendo l'apoptosi attraverso la via intrinseca.

Discussione

Il melanoma, uno dei cancri cutanei più aggressivi, la cui incidenza negli ultimi anni è notevolmente aumentata, a differenza di altre neoplasie, registra una frequenza maggiore delle diagnosi soprattutto nella fascia di popolazione più giovane. L'apoptosi rappresenta un'efficiente strategia fisiologica messa in atto dall'organismo sia durante il rimodellamento dei tessuti, sia per eliminare le cellule neoplastiche. Le cellule di melanoma, sia *in vivo* che *in vitro*, sono abbastanza refrattarie all'apoptosi [76]. È noto che un gran numero di agenti chemioterapici è in grado di indurre la morte delle cellule tumorali attraverso l'attivazione dei meccanismi apoptotici. Nel corso del dottorato di ricerca è stato valutato se il diclofenac, un farmaco anti-infiammatorio usato anche come coadiuvante chemioterapico [77], potesse esercitare un'azione citotossica in cellule di melanoma. Un nostro precedente studio, ha dimostrato che il diclofenac esercita un effetto pro-apoptotico in una linea cellulare di neuroblastoma SH-SY5Y [21] e diversi studi riportano un'azione simile da parte del diclofenac [11,15,19,78,79]. Durante il mio lavoro di dottorato, è stato evidenziato che il diclofenac esercita un effetto citotossico su due linee cellulari di melanoma umano metastatico, A2058 e SAN, mentre non provoca effetti rilevanti in una linea cellulare di fibroblasti umani immortalizzati non maligni, BJ-5ta. In particolare, è stato dimostrato che il processo apoptotico indotto nelle cellule di melanoma dal trattamento con il farmaco è associato a un incremento dei livelli di ROS intracellulari, alla diminuzione del rapporto tra Bcl-2 e Bax, al rilascio dal mitocondrio del citocromo *c*, all'aumento dei livelli di attività enzimatica delle caspasi-3 e -9, alla riduzione dei livelli proteici e di attività dell'enzima antiossidante mitocondriale SOD2 e alla sua traslocazione citosolica di quest'ultimo. Le cellule BJ-5ta sono state scelte come controllo non maligno,

invece dei melanociti, perché i fibroblasti rappresentano un tipo cellulare presente abbondantemente e in modo ubiquitario in tutti i tessuti del corpo. La valutazione del grado di tossicità di un farmaco in cellule ubiquitarie come i fibroblasti, è più informativa rispetto a quella ottenuta utilizzando cellule più rare e specializzate come i melanociti.

I cambiamenti morfologici indotti dal diclofenac nelle cellule A2058 e SAN suggeriscono che il farmaco provoca le alterazioni tipiche del processo apoptotico. L'effetto del farmaco è selettivo per le cellule di melanoma, poiché nei fibroblasti non maligni non si osservano variazioni morfologiche rilevanti. L'incremento della percentuale di nuclei ipodiploidi e l'aumento dell'attività della caspasi-3, rilevato nelle cellule di melanoma, confermano la maggiore suscettibilità di queste cellule al trattamento farmacologico. Per studiare il meccanismo cellulare coinvolto negli effetti citotossici del diclofenac, sono stati analizzati anche i profili di espressione proteica di due membri della famiglia Bcl-2, la proteina pro-apoptotico Bax e l'anti-apoptotica Bcl-2. Il coinvolgimento di Bcl-2 nello sviluppo dei processi di radio e chemio-resistenza, è ben documentato in letteratura [67,80,81] e l'aumento della sua espressione è stato associato alla resistenza all'apoptosi in diversi tipi di tumore, soprattutto melanomi [67,82]. Bax, invece, gioca un ruolo importantissimo nel processo apoptotico, perché regola la permeabilità mitocondriale. Quest'enzima è trattenuto fuori dal mitocondrio attraverso il legame con alcune proteine della famiglia Bcl-2. La diminuzione del rapporto Bcl-2/Bax nelle cellule A2058 e SAN è indicativa del coinvolgimento di tali proteine nel meccanismo d'azione del farmaco. I nostri dati, in particolare, suggeriscono che in seguito alla riduzione dei livelli di Bcl-2, nelle cellule di melanoma trattate con il diclofenac, Bax non sia più trattenuto nel citosol e può quindi traslocare nel mitocondrio.

Le cellule tumorali, solitamente, presentano livelli intracellulari di ROS maggiori rispetto a quelli delle cellule non maligne [83-85]. Queste molecole estremamente reattive, regolano

la proliferazione cellulare e contribuiscono, in modo determinante, alla trasformazione neoplastica della cellula [53]. D'altro canto, i ROS sono coinvolti anche nell'attivazione dei processi che controllano ed eliminano i cloni di cellule cancerose [54]. L'incremento dei livelli di ROS osservato in entrambe le linee cellulari di melanoma trattate con il diclofenac, potrebbe rappresentare uno tra gli eventi responsabili dell'attivazione del processo apoptotico. L'incremento precoce dei livelli di anione superossido, prodotto probabilmente nel corso dell'attività dei sistemi enzimatici coinvolti nell'azione di detossificazione del farmaco [61], potrebbe poi innescare la formazione di altri ROS. Lo squilibrio redox conseguente all'aumento dei livelli intracellulari di ROS potrebbe essere quindi responsabile dell'alterazione della funzionalità mitocondriale. È possibile ipotizzare, infatti, un disaccoppiamento tra i sistemi enzimatici che costituiscono la catena di trasporto degli elettroni, a causa della nota sensibilità ai ROS mostrata da questi complessi macromolecolari.

La SOD2, rappresentando il più importante enzima antiossidante presente nei mitocondri, contrasta e previene in modo efficace la disfunzione mitocondriale indotta dallo stress ossidativo. In diverse patologie la morte cellulare programmata è associata a livelli elevati e prolungati di ROS [86]. Il ruolo svolto dalla SOD2 nelle cellule tumorali, è comunque alquanto controverso. Elevati livelli di espressione della SOD2, sono stati rilevati in diversi tumori umani aggressivi; un aumento dell'attività enzimatica di tale proteina, è inoltre associata alla resistenza di alcuni tumori alla chemio- e radio-terapia [87-90]. La SOD2 potrebbe diventare, quindi, un nuovo target terapeutico per farmaci anti-tumorali che ne regolano negativamente, e in modo specifico i livelli. In un precedente lavoro del nostro gruppo di ricerca, è stato dimostrato che il diclofenac induce sia un aumento di ROS che una diminuzione dei livelli proteici e di attività della SOD2 nella linea cellulare di neuroblastoma SH-SY5Y [21]. La riduzione dei livelli proteici e di attività enzimatica

della SOD2, indotta dal diclofenac nelle cellule di melanoma, potrebbe contribuire a rendere le cellule più vulnerabili all'apoptosi indotta dal diclofenac. Nelle cellule neoplastiche, infatti, un incremento del metabolismo energetico comporta un aumento nella produzione di ROS, soprattutto di anione superossido; questa condizione costante di stress ossidativo cui sono sottoposte le cellule, le rende probabilmente più dipendenti all'azione dell'enzima SOD2, e di conseguenza più sensibili, rispetto alle cellule normali, a molecole capaci di regolare i livelli della SOD2 [91]. I nostri dati indicano, quindi, che la SOD2 potrebbe costituire un potenziale target terapeutico, poiché la sua inibizione contribuisce a regolare l'apoptosi nelle cellule cancerose. In alcuni casi, cellule neoplastiche che esprimono livelli elevati della SOD2 sono resistenti al danno indotto dalle radiazioni [92]. D'altro canto, altri lavori mostrano che la SOD2 può esercitare anche un ruolo anti-oncogenico. È stato dimostrato, infatti, che l'aumento di espressione della SOD2, può essere associato a una regressione del fenotipo neoplastico e del potenziale metastatico [93-96]. Questi dati, anche se apparentemente in contrasto, non sono sorprendenti, perché lo squilibrio dello stato redox cellulare può influenzare sia lo sviluppo sia la regressione di un fenotipo maligno. Gli esperimenti di silenziamento della SOD2, hanno dimostrato che l'effetto citotossico del diclofenac è nettamente amplificato in assenza di SOD2, in entrambe le linee cellulari di melanoma, e che la SOD2 avrebbe un effetto protettivo durante il trattamento. Questo risultato è in accordo con altri studi condotti su cellule di carcinoma squamoso dove l'espressione della SOD2 è stata silenziata [97].

La comparsa della SOD2 nella frazione citosolica di entrambe le linee cellulari di melanoma, trattate con il diclofenac, rappresenta un altro risultato estremamente interessante e poco documentato in letteratura. La presenza nel citosol della SOD2 potrebbe essere una causa, ma anche una conseguenza della disfunzione mitocondriale

indotta dal farmaco. Poiché la SOD2 è enzimaticamente attiva solamente nel compartimento mitocondriale [98], la presenza dell'enzima inattivo nel citosol potrebbe, influenzare l'equilibrio redox cellulare e, in seguito, amplificare l'effetto pro-apoptotico del diclofenac. È noto, infatti, che la sodio selenite o i sali biliari inducono la traslocazione citosolica della SOD2, contribuendo alla disfunzione mitocondriale [99].

Sono note, due tipi di vie di attivazione dell'apoptosi; una via estrinseca, la cui attivazione prevede il legame di alcuni fattori extracellulari con recettori cellulari "di morte" che trasmettono poi alla cellula il segnale apoptotico, ed una via intrinseca, la cui attivazione è mediata dai mitocondri. Questi organuli cellulari esercitano un ruolo fondamentale nel controllo del processo apoptotico, soprattutto se indotto da farmaci [100]. Bouchier-Hayes e colleghi [101], indentificano il mitocondrio come specifico target farmacologico. I risultati ottenuti nel corso del mio dottorato di ricerca, indicano che l'effetto pro-apoptotico del diclofenac, indotto nelle cellule di melanoma, è mediato dall'attivazione della via intrinseca dell'apoptosi. Durante questo processo, si osserva, infatti, una riduzione del rapporto Bcl2/Bax, una traslocazione dal mitocondrio al citosol del citocromo *c* e della SOD2 e un aumento significativo dell'attività della caspasi 9, evento mediato dalla traslocazione citosolica del citocromo *c* [75].

I risultati prodotti suggeriscono, quindi, un chiaro coinvolgimento del mitocondrio nel meccanismo di azione citotossico del diclofenac. L'aumento dei livelli di ROS, modulando il rapporto Bcl-2/Bax [102], induce la traslocazione nel citosol del citocromo *c*, e in seguito, l'attivazione della caspasi 9. La riduzione dei livelli della SOD2 e la comparsa della proteina nel citosol, sono indicativi di una disfunzione mitocondriale indotta dal trattamento farmacologico selettivamente nelle cellule di melanoma. Nei fibroblasti non maligni BJ-5ta, non si osservano, infatti, alterazioni significative dei parametri precedentemente descritti. Uno delle cause della minore sensibilità mostrata dai fibroblasti

verso il farmaco potrebbe essere dovuta a una minore attività dei sistemi enzimatici deputati ai processi di detossificazione farmacologica. È stato riportato, infatti, che in alcune linee cellulari di fibroblasti sono presenti livelli inferiori di questi complessi enzimatici [103,104].

Recentemente, molti studi hanno attribuito al mitocondrio un ruolo chiave nel processo di trasformazione neoplastica [85,36]. Il livello elevato di ROS rilevato nelle cellule neoplastiche potrebbe derivare dal numero maggiore di mitocondri. Ne consegue che la cellula tumorale può essere più esposta a mutazioni, evento che contribuisce ad accentuare il fenotipo cellulare maligno, permettendo quindi alla cellula di sfuggire a meccanismi di controllo del ciclo cellulare. È quindi importante identificare e studiare nuove molecole con attività anti-neoplastica, che possano attivare nel mitocondrio una massiccia produzione di ROS, tale da indurre la morte selettiva delle cellule cancerose. Questi farmaci, associati ai trattamenti chemioterapici convenzionali, potrebbero amplificarne le proprietà terapeutiche e permettere di eludere i meccanismi di chemio-resistenza; queste nuove molecole, inoltre, potrebbero costituire una strategia terapeutica alternativa se usate in combinazione con inibitori di proteine che modulano negativamente l'apoptosi, come ad esempio la SOD2.

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Iconografia

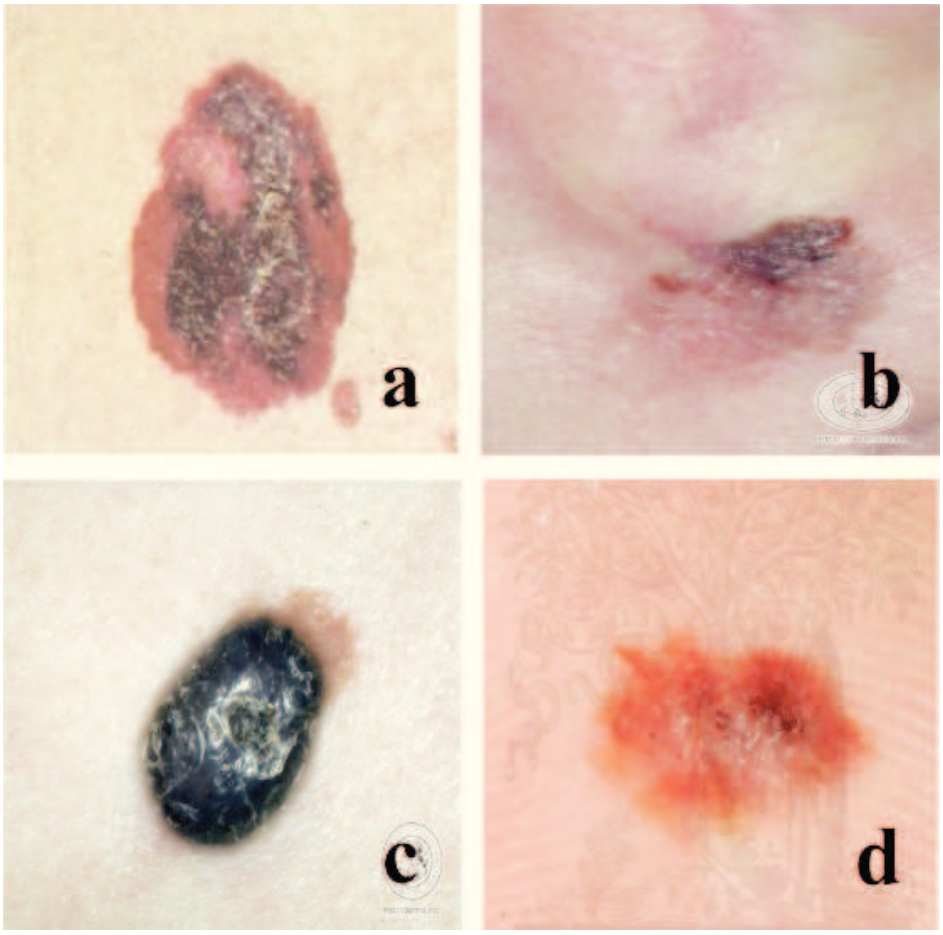

Figura 1. Melanoma a diffusione superficiale (a), lentigo maligna (b), melanoma nodulare (c), melanoma acrale (d).

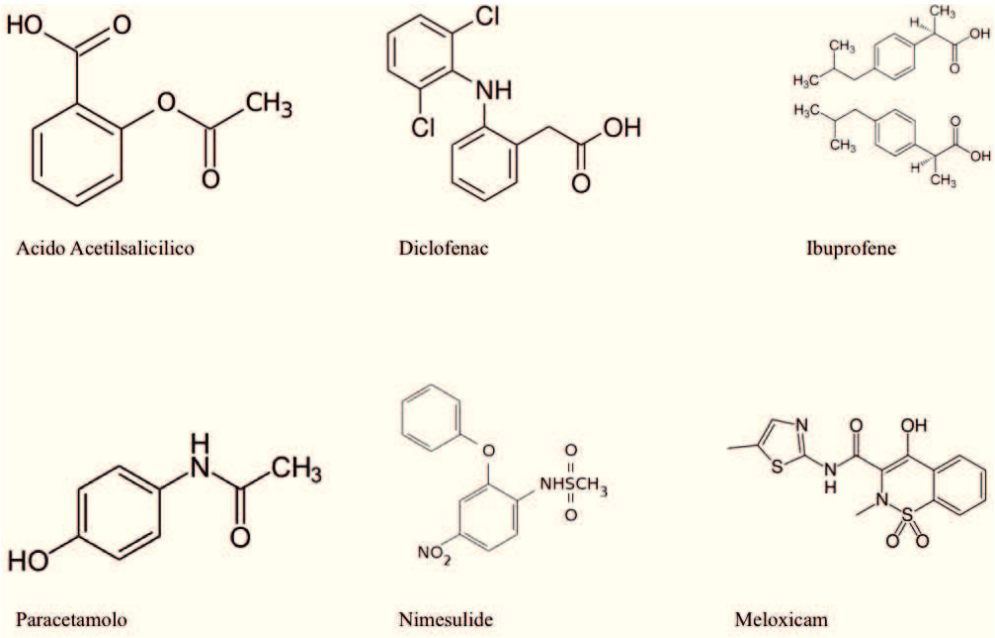

Figura 2. Struttura dei più comuni Farmaci Antinfiammatori Non Steroidei (FANS).

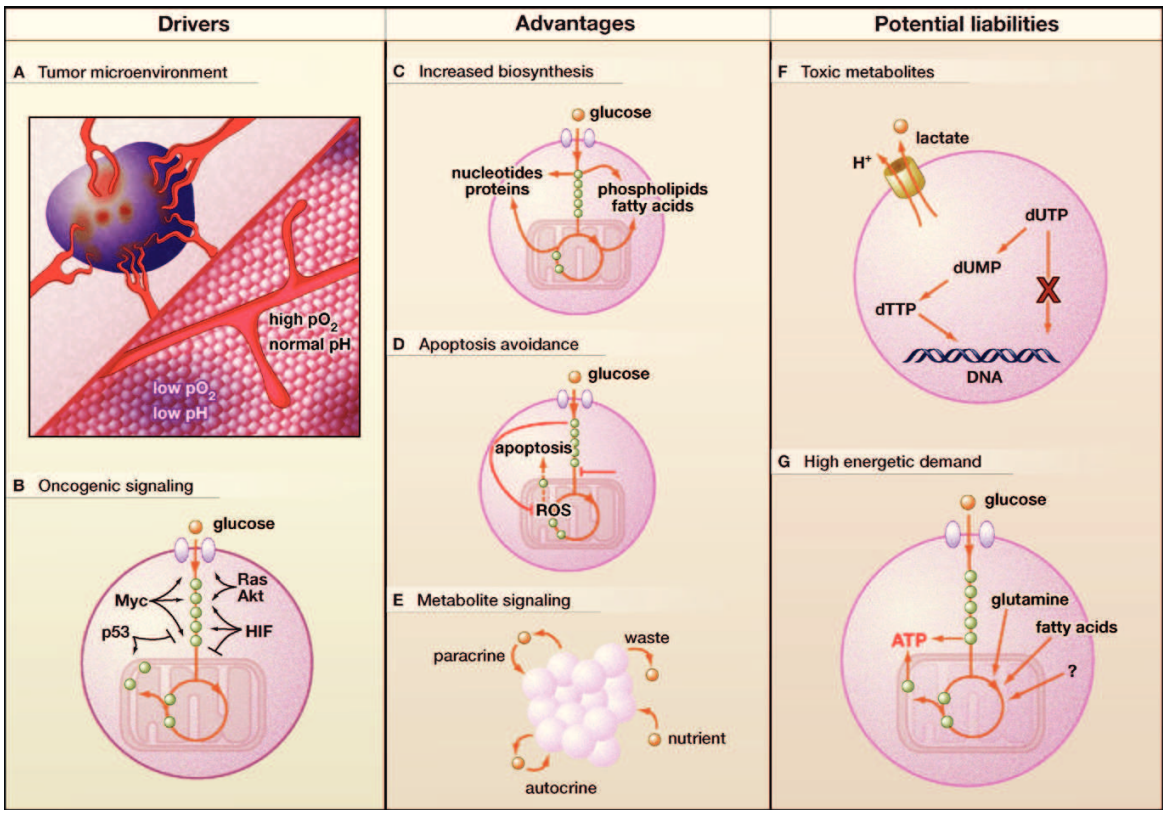

Figura 3. Il metabolismo nelle cellule tumorali.

Induttori (A e B). *A*) Il microambiente tumorale è eterogeneo, e presenta regioni con ridotte concentrazioni di ossigeno e con pH acido (zone viola). *B*) Vie di segnale coinvolte nell'alterazione metabolica delle cellule tumorali. Processi che regolano positivamente (*C*-*E*) e negativamente (*F*-*G*) lo sviluppo dei cloni neoplastici.

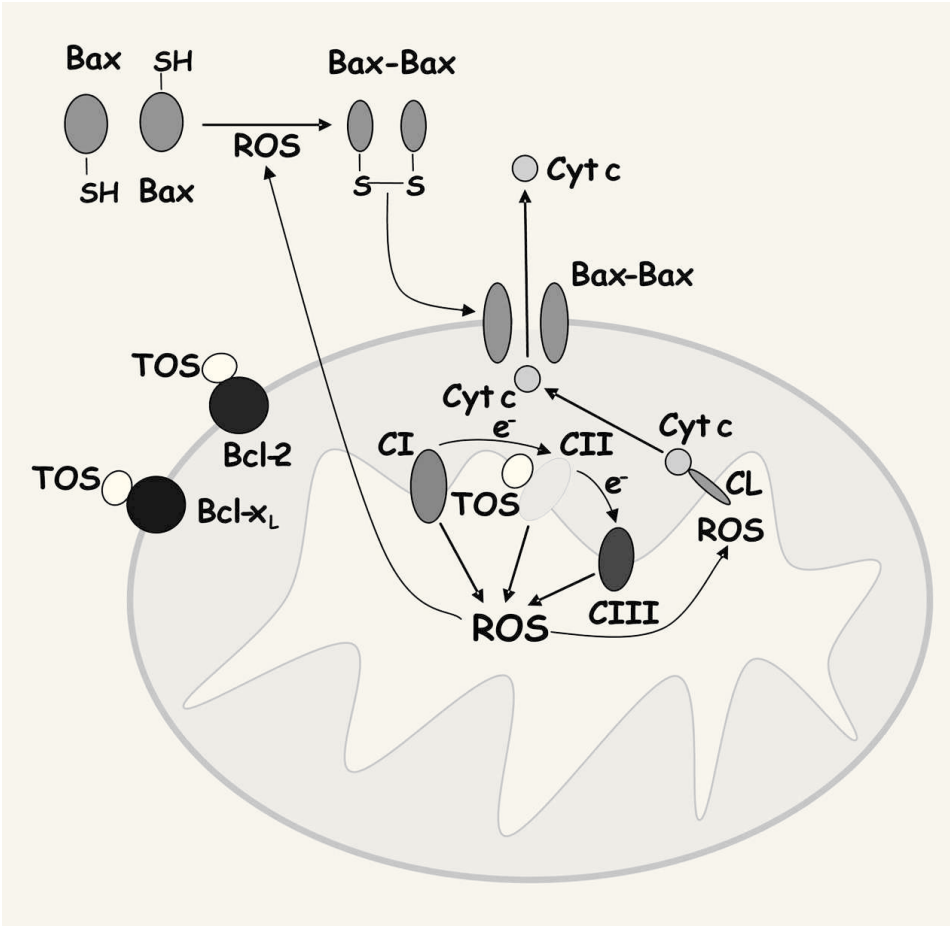

Figura 4. Fattori coinvolti nel processo proapoptotico indotto dai mitocans.

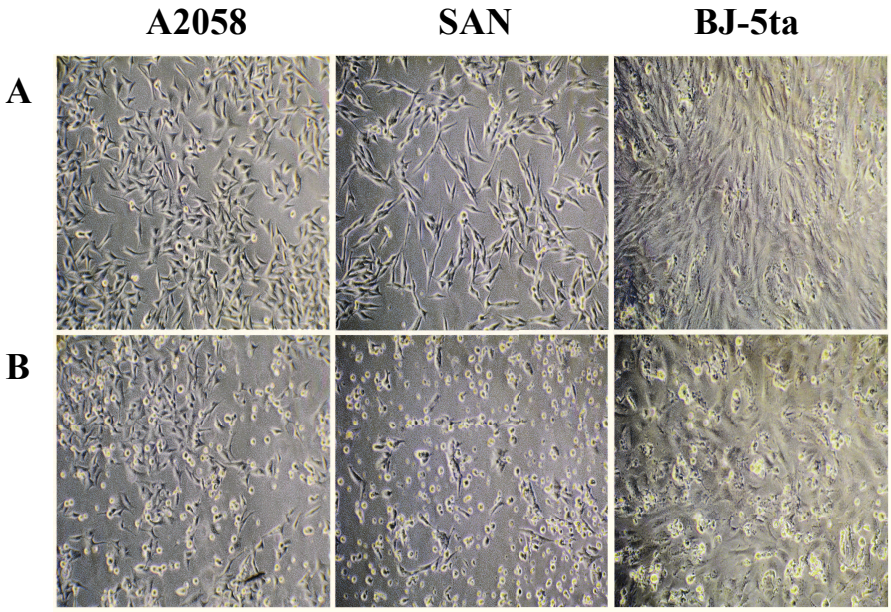

Figura 5. Alterazioni morfologiche delle cellule A2058, SAN e BJ-5ta, indotte dal trattamento con il diclofenac.

Le cellule sono state incubate per 48 ore con il solo veicolo (A) o con 150 µM di diclofenac (B). Le immagini sono rappresentative di tre esperimenti indipendenti (ingrandimento 10X).

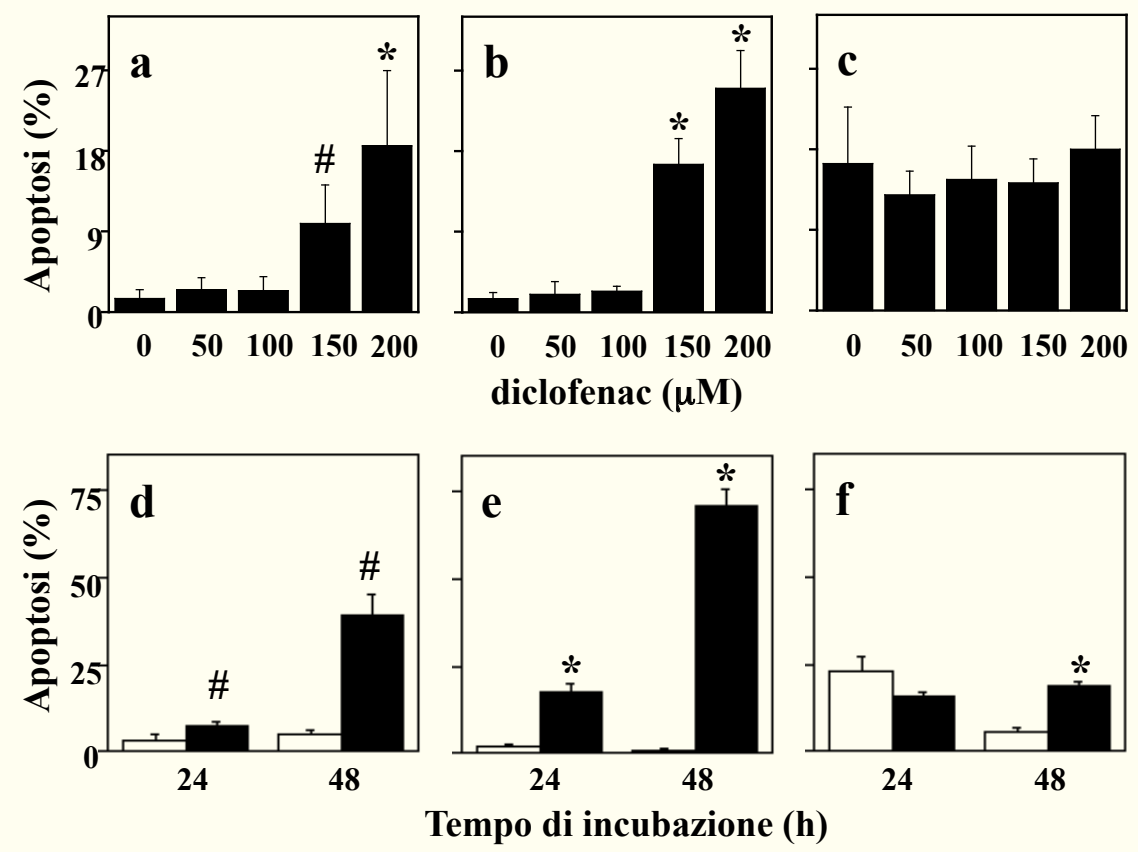

Figura 6. Valutazione dell'effetto citotossico indotto dal diclofenac nelle cellule A2058, SAN e BJ-5ta.

Effetto dose dipendente (a, b, c). Le cellule sono state incubate con le concentrazioni indicate di diclofenac e l'apoptosi è stata valutata dopo 24 ore dal trattamento con il farmaco. *Effetto tempo dipendente (d, e, f)*. Le cellule sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera). L'apoptosi è stata valutata dopo i tempi di incubazione indicati. I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ e *$P < .01$ paragonati ai controlli.

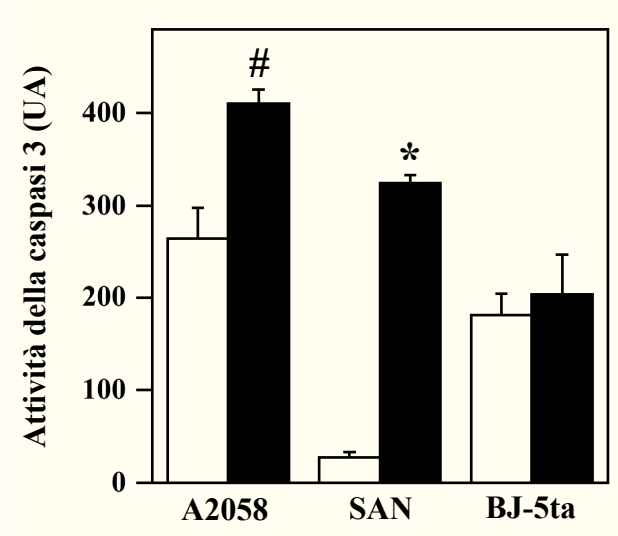

Figura 7. Effetto del diclofenac sull'attività enzimatica della caspasi 3 nelle cellule A2058, SAN e BJ-5ta. Le cellule sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera). L'attività enzimatica della caspasi 3 è riportata in Unità Arbitrarie (UA). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ e *$P < .01$ paragonati ai controlli.

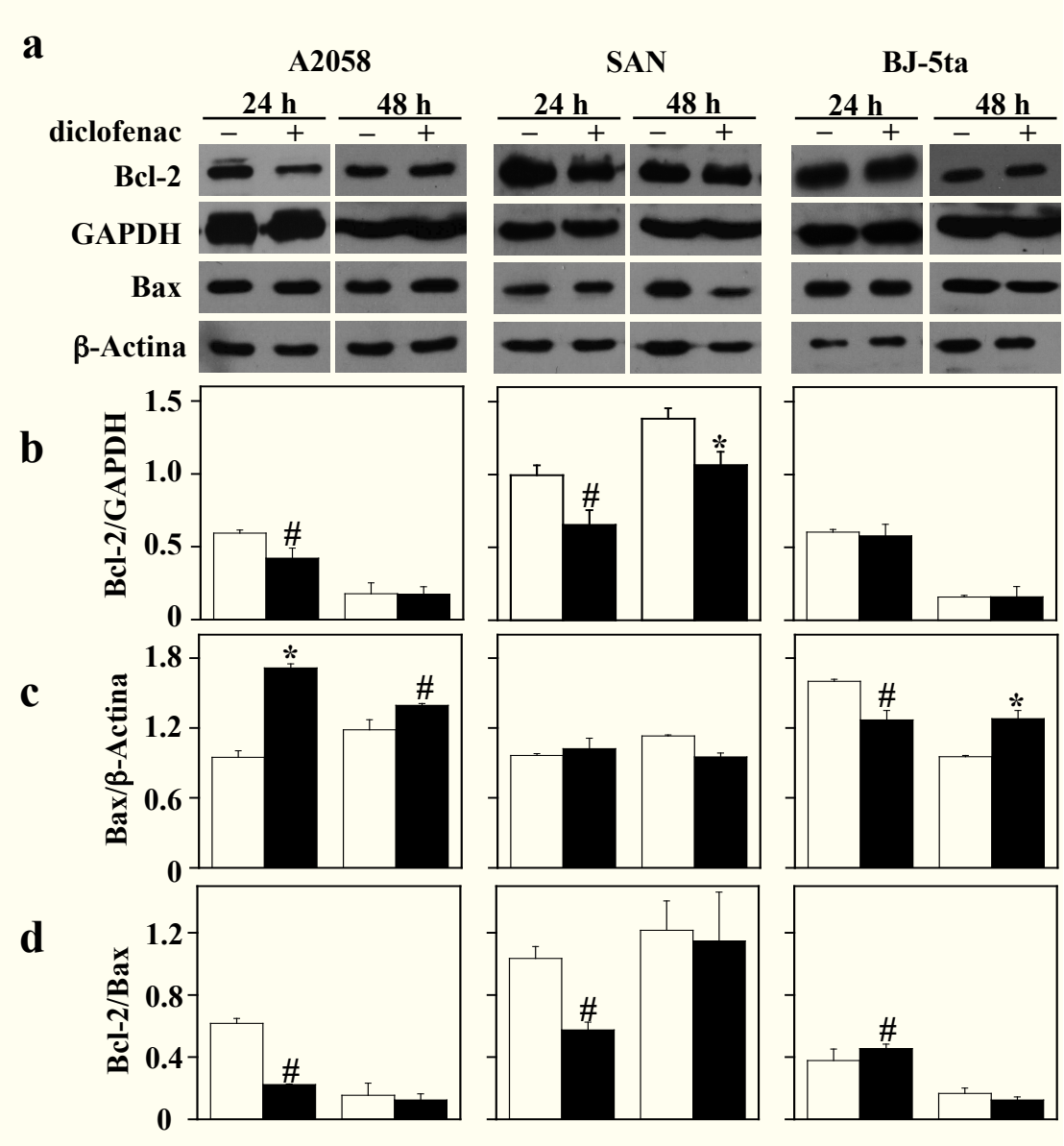

Figura 8. Effetto del diclofenac sui livelli delle proteine Bcl-2 e Bax. *Western blotting (a)*. Le cellule sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera) per 24 e 48 ore. L'analisi negli estratti cellulari totali è stata effettuata mediante Western blotting utilizzando GAPDH e β-actina come normalizzatori, rispettivamente per Bcl-2 e Bax. *Analisi densitometrica dei livelli interni delle proteine Bcl-2 e Bax (b, c)*. Determinazione del rapporto Bcl-2/Bax (d). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ e *$P < .01$ paragonati ai controlli.

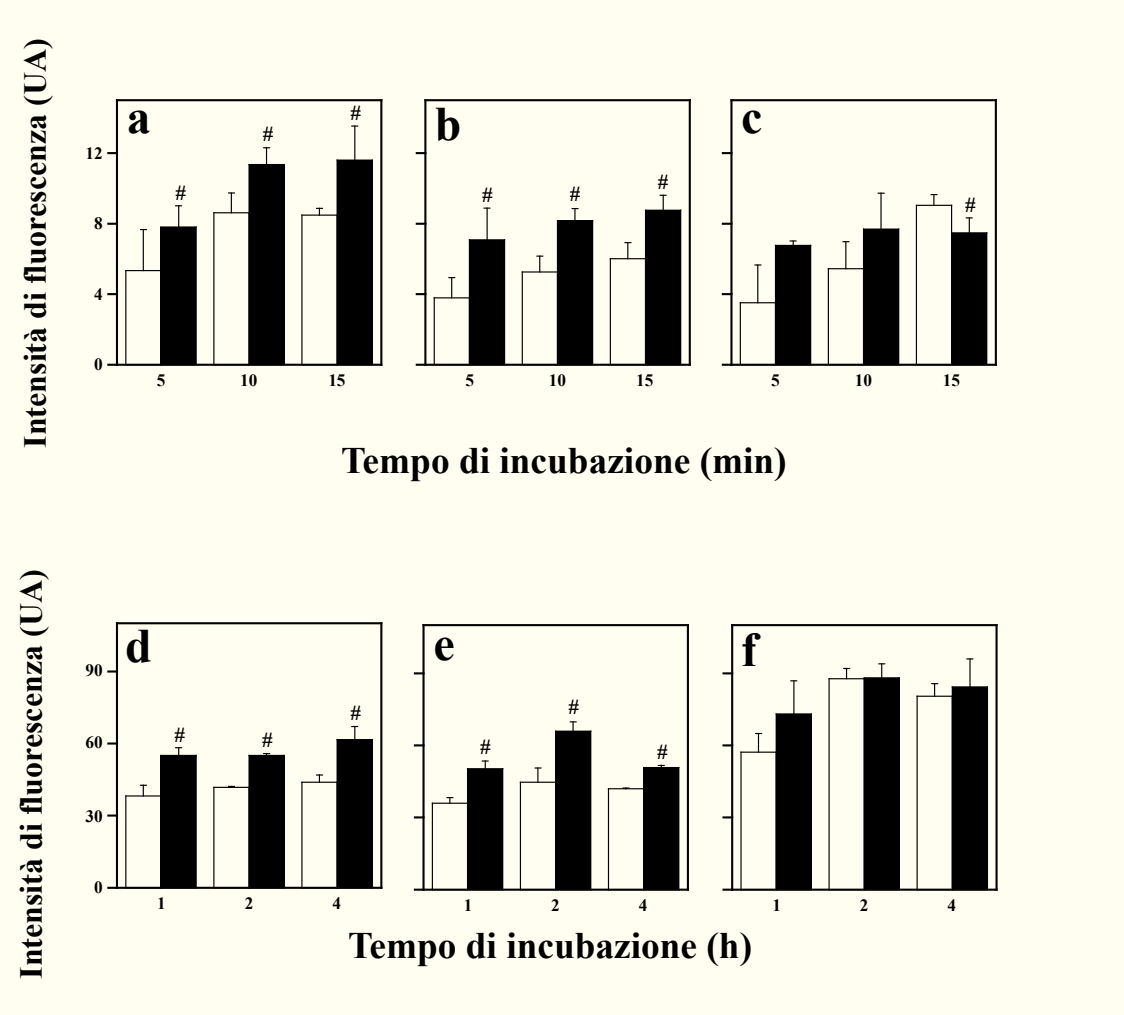

Figura 9. Effetto del diclofenac sui livelli intracellulari di ROS. Le cellule A2058 (a, d), SAN (b, e) e BJ-5ta (c, f), sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera), per i tempi indicati. I livelli intracellulari di ROS sono stati valutati utilizzando la sonda fluorescente DHE (a, b, c) o DCFH-DA (d, e, f). L'intensità di fluorescenza è riportata in Unità Arbitrarie (UA). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ paragonati ai controlli.

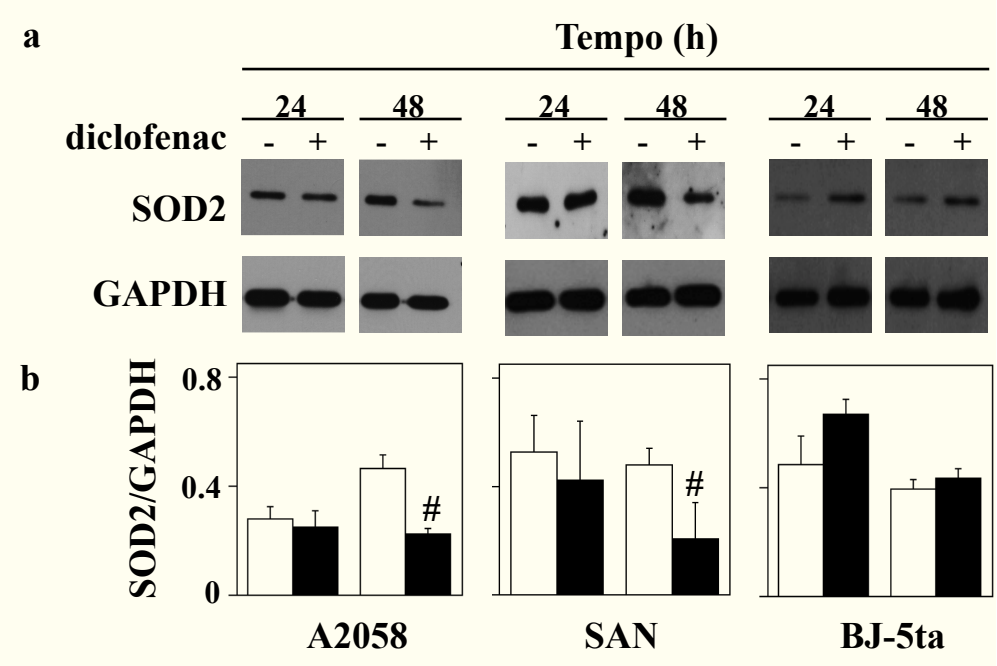

Figura 10. Effetto del diclofenac sui livelli della proteina SOD2. *Western blotting (a)*. Le cellule A2058, SAN e BJ-5ta sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera) per 24 e 48 ore. L'analisi negli estratti cellulari totali è stata effettuata mediante Western blotting utilizzando GAPDH come normalizzatore. *Analisi densitometrica dei livelli della proteina SOD2 (b)*. I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ paragonati ai controlli.

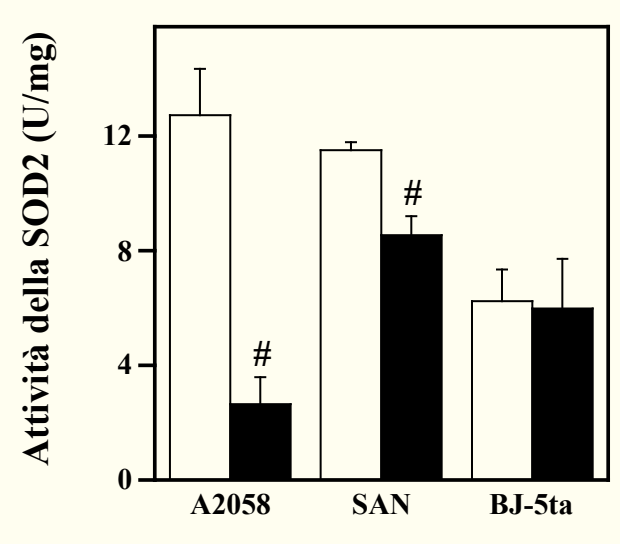

Figura 11. Effetto del diclofenac sull'attività enzimatica della proteina SOD2. Le cellule A2058, SAN e BJ-5ta sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera) per 48 ore. L'attività della SOD2 negli estratti proteici mitocondriali, è stata misurata mediante una tecnica spettrofotometrica, ed espressa come attività specifica (U/mg). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ paragonati ai controlli.

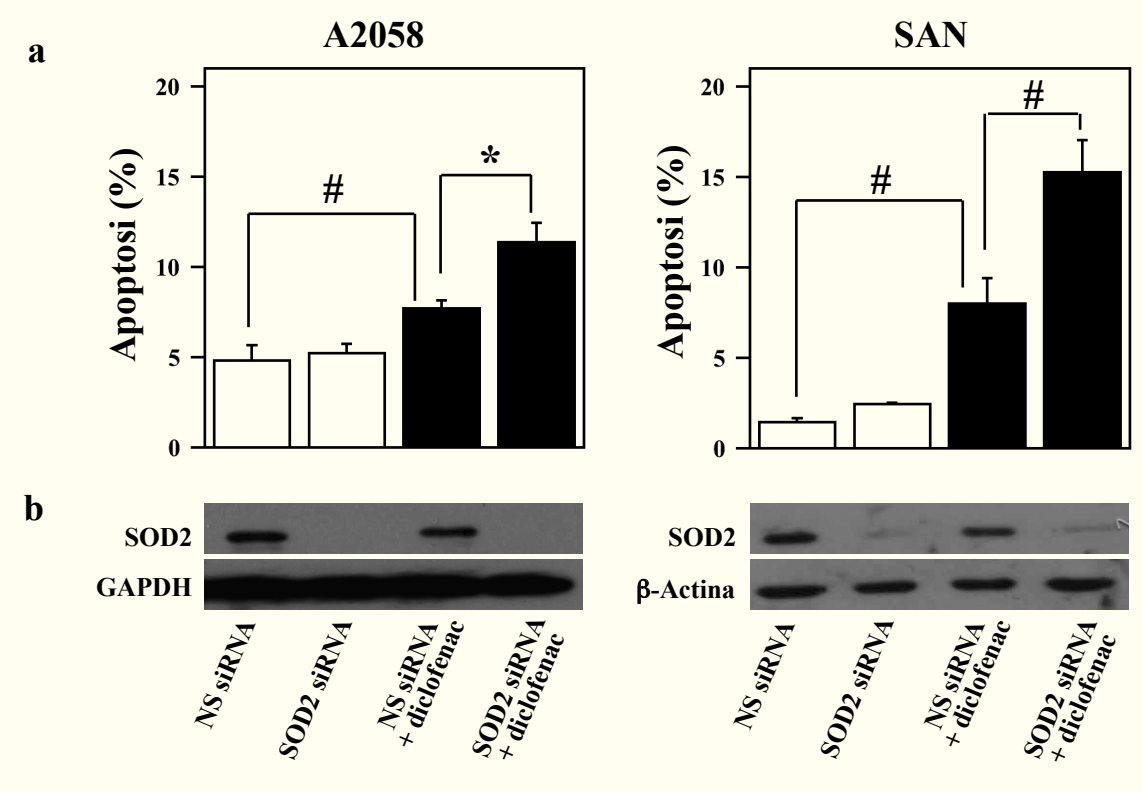

Figura 12. Amplificazione dell'effetto pro-apoptotico del diclofenac in cellule di melanoma, dopo il silenziamento del gene per la SOD2. Le cellule A2058 e SAN sono state trasfettate con NS siRNA o SOD2 siRNA e, dopo 72 ore, esse sono state incubate per ulteriori 24 ore con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera). I campioni sono stati poi divisi in due aliquote. Un'aliquota è stata usata per la valutazione dell'apoptosi tramite saggio citofluorimetrico (a). L'altra aliquota è stata usata per valutare i livelli di SOD2 mediante Western blotting, usando GAPDH e β-actina, come controllo interno, per le A2058 e le SAN, rispettivamente (b). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ e *$P < .01$ paragonati ai controlli.

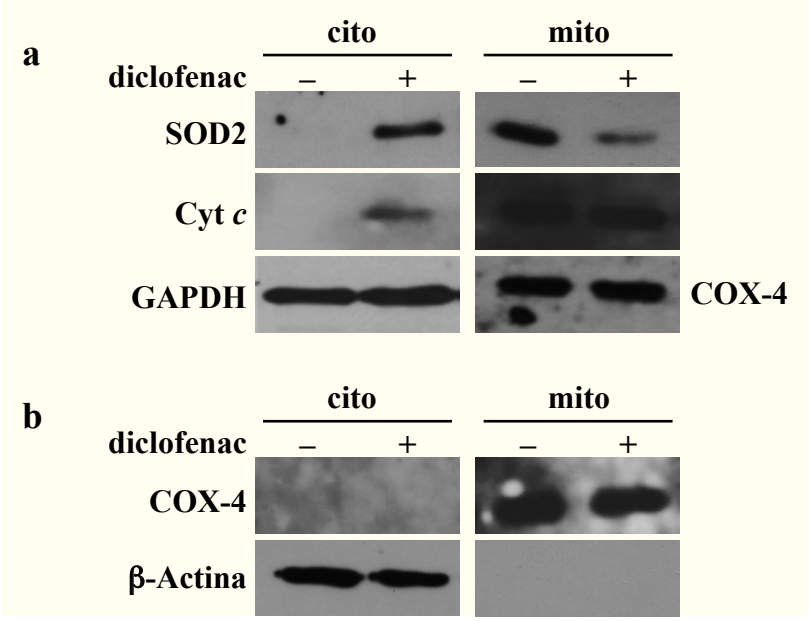

Figura 13. Effetto del diclofenac sulla localizzazione subcellullare della SOD2 e del citocromo *c* nelle cellule A2058. Le cellule sono state incubate per 24 ore, con il solo veicolo o con 150 μM di diclofenac, e successivamente sono stati preparati gli estratti proteici mitocondriali e citosolici. GAPDH e COX-4 sono stati utilizzati come controlli interni rispettivamente per la normalizzazione citosolica e mitocondriale (a). *Valutazione del grado di purezza delle frazioni subcellulari mediante Western blotting*. β-actina e COX-4 sono stati utilizzati rispettivamente come marcatori specifici del citosol e del mitocondrio (b).

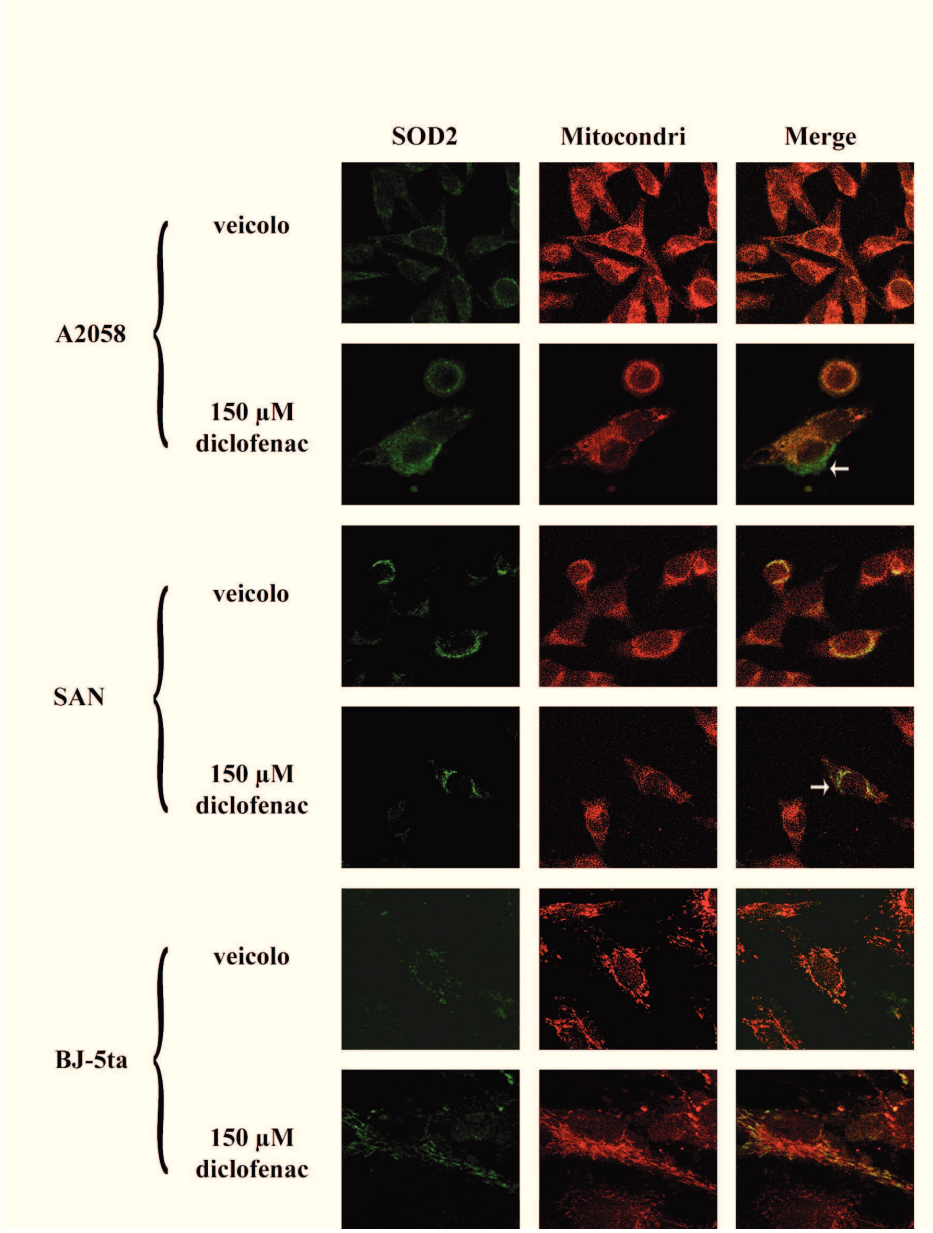

Figura 14. Immagini al microscopio confocale della localizzazione subcellulare della SOD2 in cellule di melanoma e fibroblasti durante il trattamento con il diclofenac. Le cellule A2058, SAN e BJ-5ta sono state incubate per 24 ore con il solo veicolo o con 150 µM di diclofenac. I mitocondri sono stati colorati con 90 nM di MitoTracker Red® per un'ora (fluorescenza rossa), e successivamente l'immunofluorescenza della SOD2 è stata rilevata utilizzando un anticorpo policlonale anti-SOD2, riconosciuto da un anticorpo secondario coniugato con FITC. La sovrapposizione del segnale specifico della SOD2 con MitoTracker Red® (Merge) è visibile come fluorescenza gialla. Le frecce indicano la presenza della SOD2 nel citosol (fluorescenza verde).

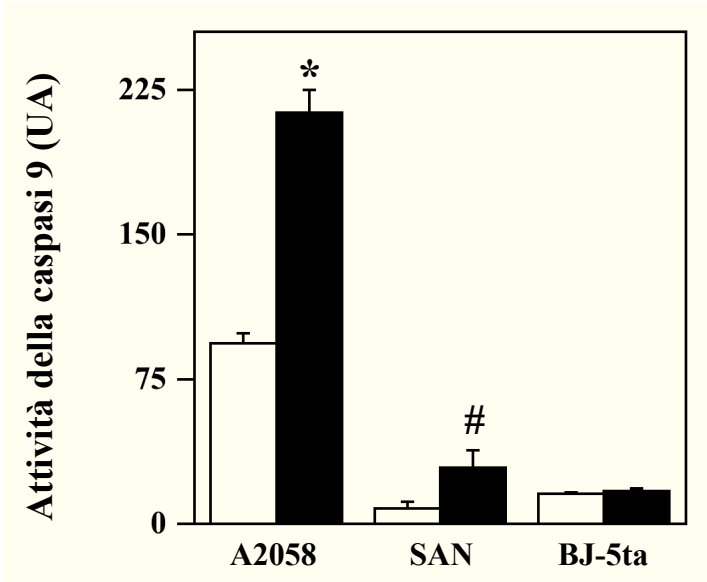

Figura 15. Effetto del diclofenac sull'attività della caspasi 9 nelle cellule A2058, SAN e BJ-5ta. Le cellule sono state trattate con il solo veicolo (barra bianca) o con 150 μM di diclofenac (barra nera). L'attività enzimatica della caspasi 9 è riportata in Unità Arbitrarie (UA). I dati sono espressi come la media di esperimenti condotti in triplicato ± SD. #$P < .05$ e *$P < .01$ paragonati ai controlli.

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Research paper

Markers of mitochondrial dysfunction during the diclofenac-induced apoptosis in melanoma cell lines

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ABSTRACT

Melanoma is an aggressive cutaneous cancer, whose incidence is growing in recent years, especially in the younger population. The favorable therapy for this neoplasm consists in its early surgical excision; otherwise, in case of late diagnosis, melanoma becomes very refractory to any conventional therapy. Nevertheless, the acute inflammatory response occurring after excision of the primary melanoma can affect the activation and/or regulation of melanoma invasion and metastasis. Nonsteroidal anti-inflammatory drugs (NSAIDs), widely employed in clinical therapy as cyclooxygenase inhibitors, also display a cytotoxic effect on some cancer cell lines; therefore, their possible usage in combination with conventional chemo- and radio-therapies of tumors is being considered. In particular, diclofenac, one of the most common NSAIDs, displays its anti-proliferative effect in many tumor lines, through an alteration of the cellular redox state. In this study, the possible anti-neoplastic potential of diclofenac on the human melanoma cell lines A2058 and SAN was investigated, and a comparison was made with the results obtained from the nonmalignant fibroblast cell line BJ-5ta. Either in A2058 or SAN, the diclofenac treatment caused typical apoptotic morphological changes, as well as an increase of the number of sub-diploid nuclei; conversely, the same treatment on BJ-5ta had only a marginal effect. The observed decrease of Bcl-2/Bax ratio and a parallel increase of caspase-3 activity confirmed the pro-apoptotic role exerted by diclofenac in melanoma cells; furthermore, the drug provoked an increase of the ROS levels, a decrease of mitochondrial superoxide dismutase (SOD2), the cytosolic translocation of both SOD2 and cytochrome *c*, and an increase of caspase-9 activity. Finally, the cytotoxic effect of diclofenac was amplified, in melanoma cells, by the silencing of SOD2. These data improve the knowledge on the effects of diclofenac and suggest that new anti-neoplastic treatments should be based on the central role of mitochondrion in cancer development; under this concern, the possible involvement of SOD2 as a novel target could be considered.

1. Introduction

Melanoma is a tumor characterized by a high degree of aggressiveness and its treatment mainly depends on the time of diagnosis. If realized in the early stage, the surgical excision of this neoplasia is successful; however, its late diagnosis leads to an unfavorable fate. Indeed, in the metastatic stage, melanoma becomes very refractory to conventional therapies and nodal metastasis can be associated with 70% mortality after 10 years [1,2]. The excision of a primary melanoma causes an acute inflammatory response during normal wound healing. This reaction prevents infections and induces cellular proliferation necessary for tissue repair. On the other hand, the same early inflammatory signals are likely involved in the activation/regulation of the melanoma invasion and metastasis. Indeed, it has been reported that pro-inflammatory cytokines upregulate integrins and the expression of adhesion molecules in a human melanoma cell line [3]; furthermore, the usage of anti-inflammatory drugs is probably associated to a lower incidence of tumor insurgence/recurrence, as for instance observed for the colorectal cancer [4–6]. Diclofenac is

Abbreviations: COX, cyclooxygenase; COX-4, cytochrome *c* oxidase IV; MPTP, mitochondrial permeability transition pore; NSAID, nonsteroidal anti-inflammatory drug; PI, propidium iodide; ROS, reactive oxygen species; SOD2, human mitochondrial manganese superoxide dismutase.

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a nonsteroidal anti-inflammatory drug (NSAID) widely used in clinical therapy. This compound inhibits cyclooxygenases (COXs), enzymes involved in the synthesis of prostaglandins, which provoke the inflammation. In the last few years, it has been reported that NSAIDs can affect negatively the development of some cancers [7,8], by inducing apoptosis and cell cycle arrest [9,10]. In particular, diclofenac has a cytotoxic effect in many cultured cell lines through an alteration of the cellular redox state [11,12]. Moreover, in a previous study we have shown that diclofenac induces apoptosis in a neuroblastoma cell line, SH–SY5Y, causing a mitochondrial dysfunction [13]. Indeed, the treatment of SH–SY5Y with diclofenac increased the intracellular level of reactive oxygen species (ROS) and reduced both protein levels and enzymatic activity of the antioxidant enzyme mitochondrial superoxide dismutase (SOD2). It is interesting to note that neuroblastoma and melanoma share a common neuroectodermal origin; another similarity between the two tumors is that both are very refractory to a conventional chemotherapy and/or radiation treatment. Therefore, the assessment of new therapeutic strategies for helping and/or replacing the actual clinical treatments of these neoplasms is of great relevance.

In this study we have analyzed the cytotoxic effect of diclofenac on melanoma cell lines. In particular, the effects of diclofenac were evaluated in two human melanoma cell lines, A2058 and SAN, and in a human immortalized fibroblastic cell line, BJ-5ta. We have analyzed the events that trigger apoptosis during diclofenac treatment and have studied the mitochondrial involvement, with particular attention to SOD2.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin G, streptomycin, trypsin were purchased from Lonza. Hygromycin B was purchased from Invitrogen. Diclofenac was obtained from Calbiochem. Medium 199, propidium iodide (PI), dichlorofluorescein diacetate (DCFH–DA) and dihydroethidium (DHE) were purchased from Sigma. A protease inhibitor cocktail was obtained from Roche Diagnostics. Caspase-3 and -9 fluorometric assay kits were purchased from BioVision. Rabbit polyclonal antibody against human SOD2 was purchased from Millipore; rabbit polyclonal antibody against Bcl-2 was from Sigma; rabbit monoclonal antibody against GAPDH was obtained from Cell Signaling; goat polyclonal antibody against β-actin, goat polyclonal antibody against cytochrome *c* oxidase IV (COX-4), mouse monoclonal antibody against cytochrome *c* and each secondary antibody conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology. All other chemicals were of analytical grade and were purchased from Sigma.

2.2. Cell culture

The human melanoma cell lines A2058, kindly provided from CEINGE, Naples (Italy) and SAN [14] were derived from lymphonodal metastasis. A2058 and SAN melanoma cells were grown in DMEM and RPMI 1640 medium, respectively, supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin in humidified incubator at 37 °C under 5% CO_2 atmosphere. BJ-5ta [15], a human skin fibroblast cell line immortalized with the human telomerase reverse transcriptase (hTERT), was cultured in a 4:1 mixture of DMEM and Medium 199 supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, 100 IU/mL penicillin G, 100 μg/mL streptomycin and .01 mg/mL hygromycin B in humidified incubator at 37 °C under 5% CO_2 atmosphere. All cells were split and seeded in plates (75 cm^2) every three days and used during the exponential phase of growth. Cell treatments were always carried after 24 h from plating.

2.3. Evaluation of apoptosis

To determine the number of apoptotic nuclei, 3×10^4 cells/well were seeded into 96-well plates; at the end of each treatment, cell suspensions were centrifuged and pellets were resuspended in a hypotonic lysis solution containing 50 μg/mL PI. After incubation at 4 °C for 30 min, cells were analyzed by flow cytometry to evaluate the presence of nuclei with a DNA content lower than the diploid [16].

2.4. Measurement of the intracellular ROS content

The intracellular ROS levels were detected using the oxidation-sensitive fluorescent probes DHE and DCFH–DA [17,18]. Briefly, cells were seeded into 6-well plate (3×10^5 cells/well) and treated with 150 μM diclofenac at different times. DHE was added in the dark at 10 μM final concentration, 30 min before the addition of the drug. DCFH–DA was added in the dark at 10 μM final concentration, 30 min before the end of each incubation with the drug; then cells were collected, washed in 10 mM sodium phosphate, pH 7.2 buffer containing 150 mM NaCl (PBS), and finally resuspended in 500 μL of PBS for the fluorimetric analysis. The measurement of the ROS levels was realized with a Cary Eclipse fluorescence spectrophotometer (Varian). Excitation and emission wavelengths were 392 nm, and 410 nm for DHE, and 485 nm and 538 nm, for DCFH–DA respectively; both excitation and emission slits were set at 10 nm.

2.5. Total cell lysates and Western blotting analysis

Cells were plated at a density of 0.3×10^6 cells/well in 6-well plates. After the treatment, cells were harvested, washed with PBS and then lysed in ice-cold modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM Na_3VO_4 and 1 mM NaF), supplemented with protease inhibitors and incubated for 30 min on ice. The supernatant obtained after centrifugation at 12,000 *g* for 30 min at 4 °C constituted the total protein extract. Protein concentration was determined by the method of Bradford [19], using bovine serum albumin (BSA) as standard. Western blotting analysis was realized on equal amounts of total protein extracts. Briefly, protein samples were dissolved in SDS-reducing loading buffer, run on a 14% SDS/PAGE and then transferred to Immobilon P membrane (Millipore). The filter was incubated with the specific primary antibody at 4 °C overnight and then with the horseradish peroxidase-linked secondary antibody at room temperature for 1 h. Membranes were then analyzed by an enhanced chemiluminescence reaction, using Super Signal West Pico kit (Pierce) according to the manufacturer's instructions; signals were visualized by autoradiography.

2.6. Measurements of caspase-3 and -9 activity

To evaluate caspase-3 and -9 activation during diclofenac treatment, the relative enzymatic activities were measured by using caspase-3 and -9 fluorometric assay kits according to the manufacturer's protocol. Briefly, cells were seeded into 75 cm^2 plates (2×10^6 cells/plate) and treated with 150 μM diclofenac. At the end of each incubation cells were collected, washed with PBS, and finally lysed at 4 °C in the cell lysis buffer. Cell lysates (150 μg) were incubated with 50 μM DEVD-AFC or LEHD-AFC substrates at 37 °C for 2 h, to detect caspase-3 or caspase-9 activity, respectively.

The measurements were realized with a Cary Eclipse fluorescence spectrophotometer (Varian). Excitation and emission wavelengths were 400 nm and 505 nm, respectively; both excitation and emission slits were set at 10 nm.

2.7. Small interference RNA

The SOD2-specific small interfering RNA (SOD2 siRNA) and non-silencing scrambled siRNA (NS siRNA) were purchased from Dharmacon. Cell transfection with siRNA was performed using Metafectene SI reagent according to the manufacturer's protocol. Briefly, cells were seeded into 12 wells/plate (0.6×10^5 cells/well) in complete medium and grown overnight. Then, 4 μL Metafectene SI and 70 nM siRNA were added to 60 μL SI buffer, left at room temperature for 15 min; the mixture was then added to the well. After 72 h, the culture medium was replaced by a new complete medium and cells were stimulated without or with 150 μM diclofenac for additional 24 h. At the end of drug treatment, cells were harvested and divided in two aliquots, to verify the SOD2 silencing by Western blotting, and to measure the apoptosis by the PI incorporation method.

2.8. Subcellular fractionation for Western blotting analysis

Cells were plated at a density of 2×10^6 cells/plate (75 cm^2). After the treatment, cells were harvested, washed in PBS and then resuspended in buffer M (5 mM Hepes, pH 7.4, 250 mM mannitol, 0.5 mM EGTA, 0.1% BSA), supplemented with protease inhibitors, and homogenized. The homogenate was centrifuged at 800 *g* for 10 min at 4 °C and the supernatant was then centrifuged at 12,000 *g* for 30 min at 4 °C. The resulting supernatant represented the cytosolic fraction, whereas the pellet, constituting the mitochondrial fraction, was resuspended in RIPA buffer. Protein concentration was determined as previously indicated. Aliquots of both fractions (cytosolic and mitochondrial) were used in Western blotting analysis.

2.9. Measurements of SOD2 activity

Cells were plated at a density of 2×10^6 cells/plate (75 cm^2). After the treatment, cells were collected and centrifuged at 300 *g* at 4 °C for 5 min, washed once with PBS and the mitochondrial fraction was prepared as described in the previous paragraph, with the following modification. The mitochondrial pellet was resuspended in 50 mM potassium phosphate, pH 7.8, containing 1 mM EDTA, sonicated, centrifuged at 20,000 *g* at 4 °C for 30 min and the resulting supernatant, after protein quantitation, was used for SOD2 assay. SOD activity was measured at 25 °C in 100 mM potassium phosphate, pH 7.8, 0.1 mM Na_2EDTA by the inhibition of cytochrome *c* reduction caused by superoxide anions generated with the xanthine/xanthine oxidase method [20,21]. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of cytochrome *c* reduction.

2.10. Immunofluorescence staining

Cells were plated on coverslips at a density of 0.3×10^6 cells/well in 6-well plates. At the end of drug treatment, cells were incubated with 90 nM MitoTracker Red (Invitrogen) at 37 °C for 1 h and then washed three times with ice-cold PBS. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked in donkey serum (Millipore) diluted 1:10 in PBS, for 60 min at room temperature. Coverslips were incubated for 1 h at 37 °C with a primary rabbit polyclonal antibody against SOD2, diluted 1:50 in PBS, and subsequently incubated for 1 h at 37 °C with a FITC donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Europe Ltd.), diluted 1:50 in PBS. Slide mounting was done in Vectashield. Fluorescent-labeled cells were viewed with a confocal laser scanner microscope Zeiss LSM 510 and the objective used was planopo 63X. The laser line was set at 488 nm for the FITC-conjugated antibody and at 543 nm for MitoTracker Red. Images were acquired simultaneously in the green and red channels, and processed using LSM software.

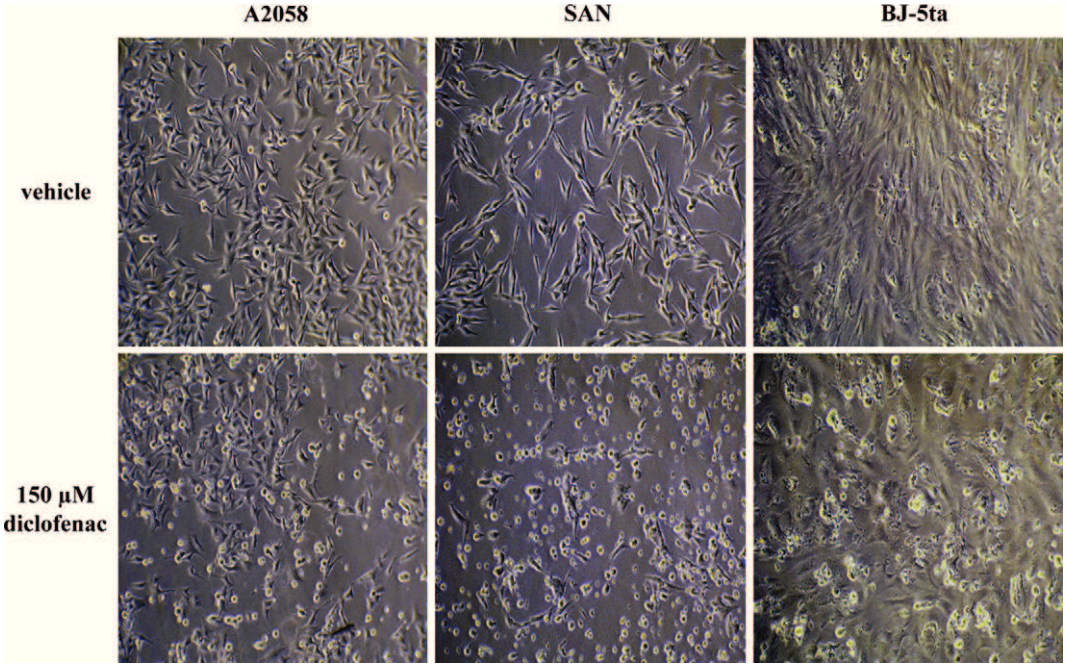

Fig. 1. Morphological changes of A2058, SAN and BJ-5ta cell lines induced by diclofenac treatment. Cells were incubated for 48 h with vehicle alone or 150 μM diclofenac. Images are representative of three independent experiments. Magnification ×10.

2.11. Statistical analysis

Numerical data were analyzed by Student's *t* test, and one-way ANOVA with Bonferroni corrections was used for multiple comparisons.

3. Results

3.1. Morphological changes of SAN, A2058 and BJ-5ta cell lines caused by diclofenac

A2058, SAN, and BJ-5ta cell lines were incubated with vehicle alone or with a diclofenac concentration ranging between 50 μM and 200 μM, and then analyzed after 24 or 48 h by light microscopy to evaluate morphological changes. Fig. 1 reports the images obtained from cells incubated with 150 μM diclofenac for 48 h. Either in A2058 or in SAN, the diclofenac treatment provoked morphological changes, such as nuclear condensation, membrane budding and cell shrinkage, that represent typical signals of the apoptotic process [22]. Interestingly, all these modifications were already evident on cells incubated with 150 μM diclofenac for 24 h or with a lower drug concentration (100 μM) for 48 h (not shown). On the other hand, in the BJ-5ta cell line (Fig. 1) these morphological changes were much less evident. Therefore, these data could suggest that the melanoma cell lines are more sensitive to the pro-apoptotic potential of diclofenac with respect to a nonmalignant fibroblast cell line.

3.2. Induction of the apoptotic process by diclofenac in melanoma cell lines

To verify whether the above-mentioned morphological changes induced by diclofenac were indicative of an apoptotic process, this program of cell death was evaluated by different techniques. In a first approach, the PI incorporation followed by flow cytometric analysis was carried out. To this aim, A2058, SAN and BJ-5ta cell lines were incubated for 24 h with an increasing concentration of diclofenac. The cytometric analysis showed a dose-dependent increase of apoptotic nuclei in A2058 (Fig. 2a) and in SAN (Fig. 2b), whereas in BJ-5ta the apoptosis induction was not observed, even in the presence of 200 μM diclofenac (Fig. 2c). To evaluate the time-course of diclofenac-induced apoptosis, cells were incubated with 150 μM diclofenac, that represents the lowest diclofenac concentration inducing apoptosis in the melanoma cell lines. After 24-h treatment, the number of nuclei with sub-diploid DNA content increased both in A2058 (Fig. 2d) and SAN (Fig. 2e), although to a different extent; on the other hand, no effect was observed in the fibroblast cell line BJ-5ta (Fig. 2f). After 48 h of drug treatment, the apoptotic process became more pronounced; in particular, in A2058, the percentage of apoptosis reached 39% with an 8-fold enhancement compared to untreated cells (Fig. 2d); in the same conditions, SAN cells were more responsive, showing 71% apoptosis (Fig. 2e). Concerning BJ-5ta, the increase in the percentage of apoptosis induced by the 48-h treatment with diclofenac was only 18%, with a modest 3-fold enhancement over untreated cells (Fig. 2f).

The pro-apoptotic effect of diclofenac was also evaluated by measuring the activity of caspase-3, the most important molecule involved in the execution of apoptosis. In fact, when caspase-3 is activated, the program for cell death becomes irreversible [23]. Hence, we have measured the caspase-3 activity in A2058, SAN and BJ-5ta cell lines treated with 150 μM diclofenac for 24 h. The data reported in Fig. 3 demonstrate that diclofenac induces an increase of caspase-3 activity in both A2058 and SAN melanoma cell lines, whereas no effect was detected for BJ-5ta. Also in this case the

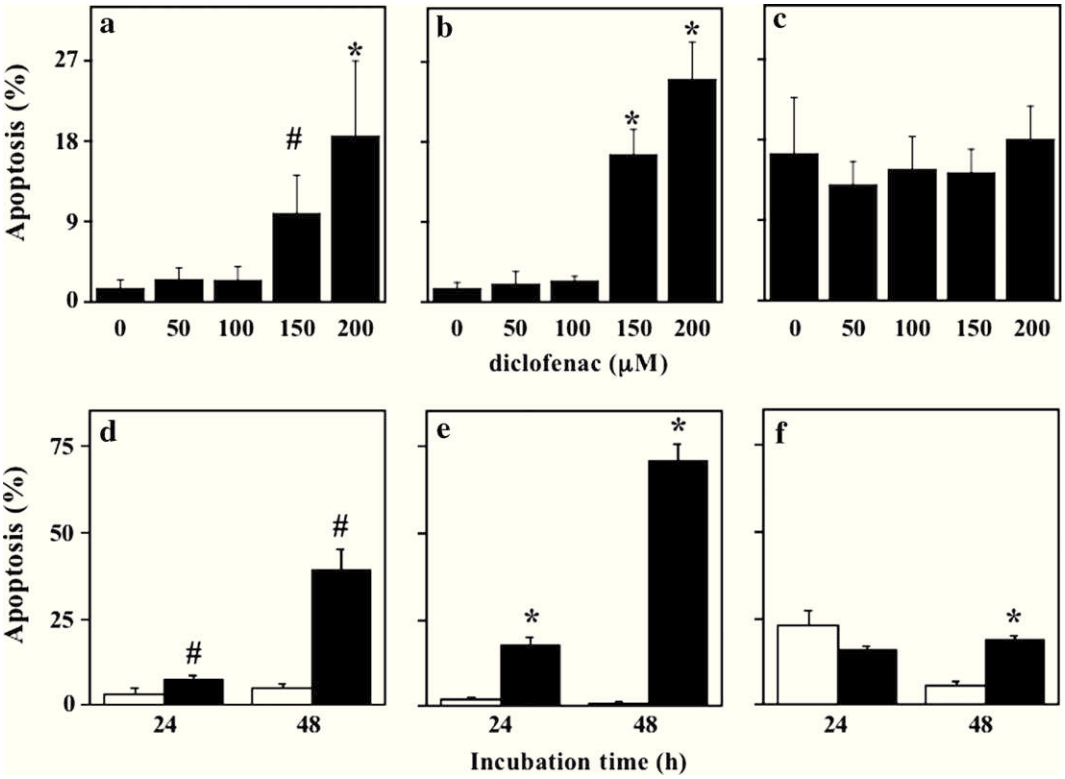

Fig. 2. Cytotoxicity of diclofenac toward A2058, SAN and BJ-5ta cell lines. (a, b, c) Dose-dependent induction of apoptosis by diclofenac. A2058 (a), SAN (b) and BJ-5ta (c) cell lines were incubated with the indicated concentrations of diclofenac and apoptosis was evaluated after a 24-h drug treatment. (d, e, f) Time-course of apoptosis by 150 μM diclofenac. A2058 (d), SAN (e) and BJ-5ta (f) cell lines were treated with vehicle alone (white bars) or 150 μM diclofenac (black bars); apoptosis was evaluated after the indicated incubation time and reported as a percentage. Data from triplicate experiments are reported as mean ± SD. $^{\#}P < .05$ and $^{*}P < .01$ compared to untreated cells.

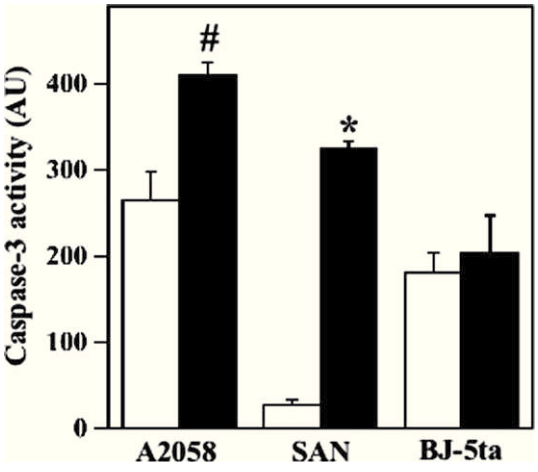

Fig. 3. Effect of diclofenac on the caspase-3 activity in A2058, SAN and BJ-5ta cell lines. Cells were incubated for 24 h with vehicle alone (white bars) or 150 μM diclofenac (black bars); caspase-3 enzymatic activity was reported as Arbitrary Units (AU). Data from triplicate experiments are reported as mean ± SD. $^{\#}P < .05$ and $^{*}P < .01$ compared to untreated cells.

responsiveness of SAN was greater than that of A2058, thanks to an initial lower level of this enzyme activity in untreated SAN cells.

To further investigate on the pro-apoptotic effect by diclofenac, we have analyzed the protein levels of two members of Bcl-2 family, the anti-apoptotic Bcl-2 and the pro-apoptotic Bax [24,25], and in particular we have evaluated the Bcl-2/Bax ratio, that regulates the apoptotic process [26]. When A2058, SAN and BJ-5ta cell lines were incubated with 150 μM diclofenac, a decrease in the Bcl-2 protein levels was observed in both melanoma cell lines after 24 h; this reduction remained detectable in SAN cells even after 48 h of incubation (Fig. 4a, b). On the contrary, in nonmalignant fibroblast cells BJ-5ta the Bcl-2 levels remained unchanged (Fig. 4a, b). A different behavior was observed for the Bax protein; indeed, its level increased in A2058 after 24-h and 48-h treatment with diclofenac, whereas it remained unchanged in SAN; concerning BJ-5ta, Bax decreased at 24 h and increased at 48 h (Fig. 4a, c). However, the Bax level was better analyzed in comparison with that of Bcl-2. For this reason, the ratio Bcl-2/Bax was measured and, as shown in Fig. 4d, a reduction of this value was evident after 24-h drug treatment in both A2058 and SAN. On the other hand, a small, although significative increase of this ratio was evident after 24 h in BJ-5ta fibroblast. Vice versa, no variation of the Bcl-2/Bax ratio was observed after 48 h in all cell lines.

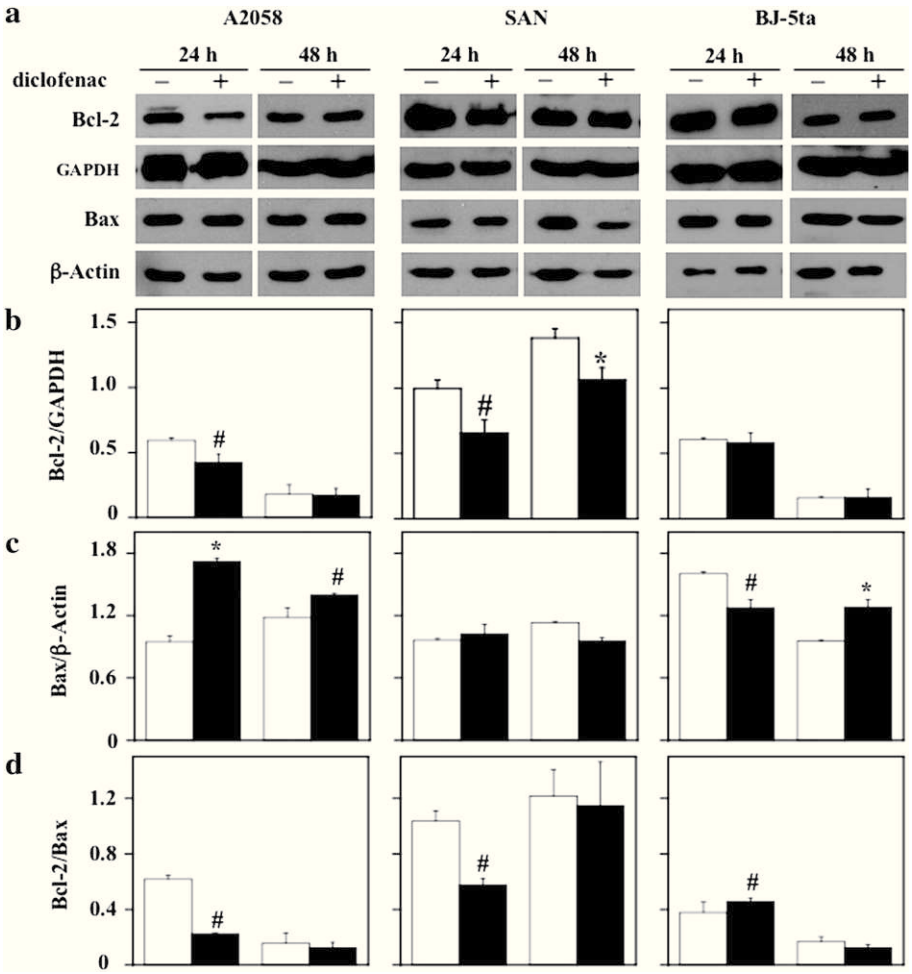

Fig. 4. Effect of diclofenac on Bcl-2 and Bax protein levels of A2058, SAN and BJ-5ta cell lines. Cells were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for 24 and 48 h. (a) Western blotting analysis was performed on total cellular extracts, using GAPDH and β-actin as loading control for Bcl-2 and Bax protein levels evaluation, respectively. (b, c) Densitometric analysis of Bcl-2 and Bax protein levels. (d) Detemination of Bcl-2/Bax ratio. Data from three independent experiments are reported as mean ± SD. $^{\#}P < .05$ and $^{*}P < .01$ compared to untreated cells.

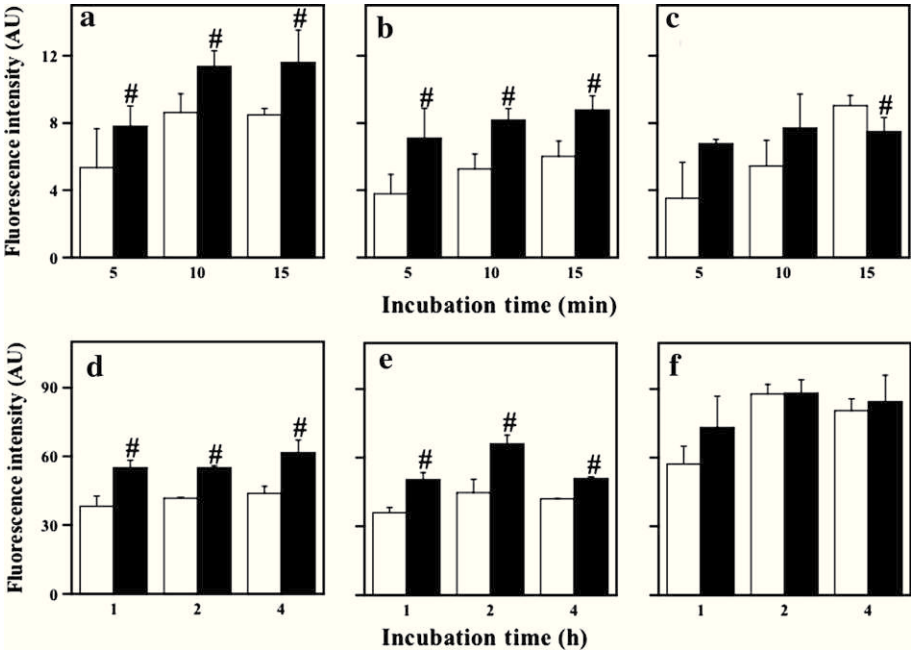

Fig. 5. Effect of diclofenac on the intracellular ROS level. A2058 (a, d), SAN (b, e) and BJ-5ta (c, f) cell lines were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for the indicated times. The intracellular ROS level was evaluated through the usage of the fluorescent probes DHE (a, b, c) or DCFH–DA (d, e, f). Fluorescence intensity was reported as Arbitrary Units (AU). Data from triplicate experiments are reported as mean ± SD. $^{\#}P < .05$ compared to untreated cells.

All these data confirm that the cytotoxic effect of diclofenac must be ascribed to a pro-apoptotic process, mainly occurring in the malignant melanoma cells.

3.3. Analysis of the intracellular ROS levels during diclofenac treatment

It is known that NSAIDs affect the redox state of different cell types [12,13,18]. Therefore, to get an insight on the mechanism of diclofenac-induced apoptosis, we have measured, during cellular drug treatment, the intracellular ROS production using the fluorescent probes DHE, a probe that mainly detects the superoxide anions, and DCFH–DA, a probe used for the evaluation of hydrogen peroxide and other superoxide-derived ROS (Fig. 5). In particular, when A2058 (Fig. 5a), SAN (Fig. 5b) and BJ-5ta (Fig. 5c) cells were incubated with diclofenac and DHE at different times, a very early increase of fluorescence intensity was observed only in the melanoma cell lines. On the contrary, in the fibroblast BJ-5ta, the drug did not cause any significant increase of superoxide levels; rather, a reduction of this radical species was evident after 15 min incubation with diclofenac. Concerning the measurements of the intracellular ROS levels with DCFH–DA, an increment of fluorescence intensity became evident only upon a longer incubation with diclofenac in both A2058 (Fig. 5d) and SAN (Fig. 5e) cells;

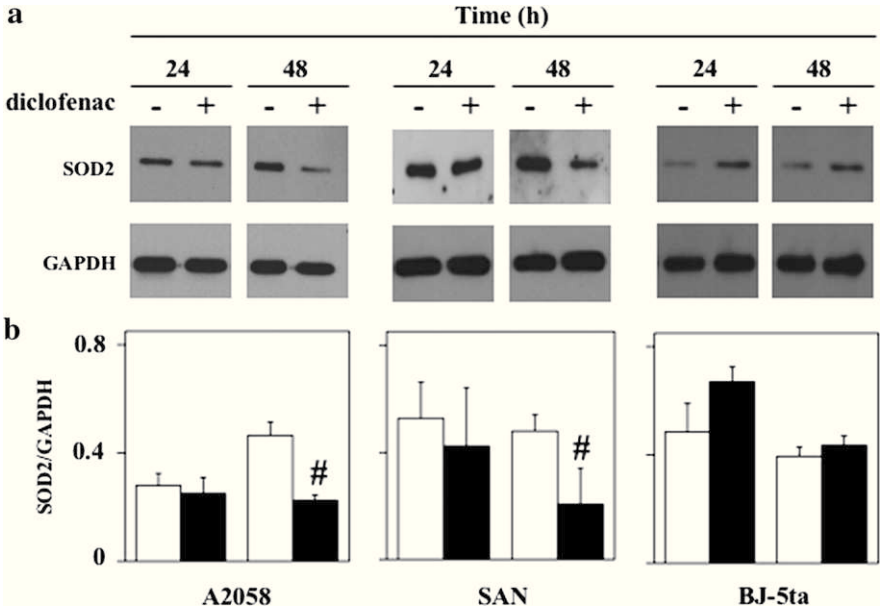

Fig. 6. Effect of diclofenac on SOD2 protein levels of A2058, SAN and BJ-5ta. Cells were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for 24 h and 48 h. (a) Western blotting analysis was performed on total cellular extracts, using GAPDH as loading control. (b) Densitometric evaluation of three independent experiments reported as mean ± SD. $^{\#}P < .05$ compared to untreated cells.

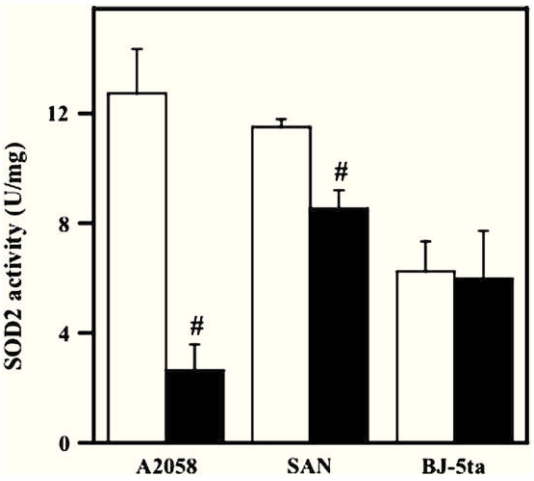

Fig. 7. Effect of diclofenac on SOD2 activity. A2058, SAN and BJ-5TA cell lines were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for 48 h. SOD2 activity was measured in mitochondrial extracts through a spectrophotometric method and reported as specific activity (U/mg). Data from triplicate experiments are reported as mean ± SD. $^{\#}P < .05$ compared to untreated cells.

conversely, no increment of ROS levels was detectable during the diclofenac treatment of BJ-5ta (Fig. 5f).

3.4. SOD2 levels during the diclofenac treatment

It is known that the mitochondrial SOD2 is the primary antioxidant enzyme, protecting cells from oxidative injuries [27]. In a previous work we showed that diclofenac caused a decrease of SOD2 protein levels in the neuroblastoma cell line SH–SY5Y [13]. Therefore, we have evaluated if diclofenac affected the SOD2 levels in SAN and A2058 cells, even considering the common neuroectodermal origin of melanoma and neuroblastoma cell lines. Fig. 6 shows the SOD2 protein levels in the melanoma and nonmalignant fibroblast cell lines treated with 150 μM diclofenac for 24 and 48 h. In both melanoma cell lines a significant reduction of the SOD2 protein level was evident after the 48-h treatment. On the other hand, the diclofenac treatment didn't cause any significant effect on SOD2 levels in BJ-5ta cells. Concerning the behavior of the Cu/Zn-SOD (SOD1), no significant alteration of its protein level was observed in A2058 cells treated with 150 μM diclofenac (not shown).

We have also evaluated if the reduction of SOD2 protein levels during the diclofenac treatment was associated to a decrease of enzyme activity in the melanoma cells. To this aim, mitochondrial protein extracts were prepared from A2058, SAN and BJ-5ta cells and incubated for 24 and 48 h in the absence or in the presence of 150 μM diclofenac. The values of SOD2 activity obtained after the 48-h treatment are shown in Fig. 7 and the data point to a significant reduction of SOD2 activity in the extracts of both melanoma cell lines treated with diclofenac; conversely, no reduction of SOD2 activity was observed in the extracts from BJ-5ta. The differences in the reduction of activity between A2058 and SAN probably reflect the different responsiveness of both melanoma cells and are apparently in line with the previous data on SOD2 protein levels. Concerning the data on the 24-h treatment, a slight reduction of SOD2 activity was hardly observed only in the mitochondrial extract from A2058 cells (not shown).

To further investigate if the reduction of SOD2 contributed to the pro-apoptotic effect of diclofenac, the A2058 and SAN melanoma cell lines were transfected with siRNAs specific for the SOD2 messenger. As shown in Fig. 8, the SOD2 silencing did not cause any significant induction of the apoptosis in the absence of diclofenac. On the other hand, in the presence of this drug, the SOD2 silencing amplified the pro-apoptotic effect of diclofenac in both melanoma

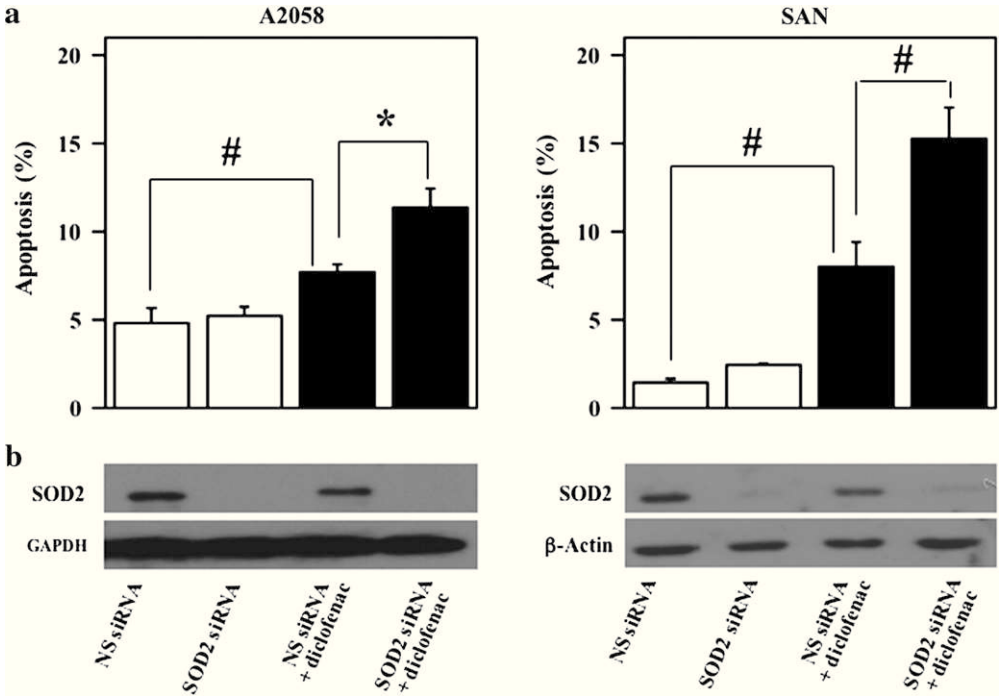

Fig. 8. Amplification of the pro-apoptotic effect of diclofenac by SOD2 gene silencing in melanoma cells. A2058 and SAN were transfected with NS siRNA or SOD2 siRNA and, after 72 h from transfection, cells were incubated for additional 24 h with vehicle alone (white bars) or 150 μM diclofenac (black bars). Each sample was divided in two aliquots. (a) One aliquot was used for the evaluation of the apoptosis by flow cytometry. The data from triplicate experiments were reported as a mean percentage ± SD; $^{\#}P < .05$ and $^{*}P < .01$ indicate the statistical relevance of the comparisons. (b) Another aliquot was used for the evaluation of SOD2 protein levels by Western blotting analysis, using GAPDH and β-actin as loading controls of total protein extracts from A2058 or SAN cells, respectively.

cell lines. These findings could suggest that SOD2 plays a relevant role in counteracting the pro-apoptotic effect of diclofenac, but is probably dispensable when these stimuli are missing.

3.5. Effect of diclofenac on subcellular distribution of SOD2 and cytochrome c, and activation of caspase-9

To better investigate on the mechanism by which diclofenac induces apoptosis in melanoma cell lines, we have evaluated the mitochondria involvement in this process, through the analysis of the localization of some typical mitochondrial proteins, such as cytochrome *c* and SOD2 (Fig. 9). Concerning cytochrome *c*, this small protein is localized in the mitochondria and, upon some death stimuli, translocates to cytosol, leading to caspase-9 activation [28,29]. Indeed, when A2058 cells were incubated with 150 μM diclofenac for 24 h, cytochrome *c* translocated from mitochondria to cytosol (Fig. 9a).

Regarding SOD2, this enzyme is considered as a typical mitochondrial marker [30,31], whereas its translocation from mitochondria to cytosol is less documented. Surprisingly, in A2058 cells the diclofenac treatment altered the subcellular distribution of SOD2, as clearly shown in Fig. 9a. Indeed, a significant amount of SOD2 appeared in the cytosolic fraction at the expenses of an almost corresponding reduction of this enzyme in the mitochondrial fraction. An identical behavior was observed upon treatment of SAN cells with diclofenac, whereas no cytochrome *c* or SOD2 translocation occurred in BJ-5ta (not shown).

To further confirm the SOD2 cytosolic translocation after the diclofenac treatment of melanoma cells, an immunofluorescence procedure was used. As shown in Fig. 10, SOD2 localization was followed by green fluorescence due to the secondary FITC antibody, whereas mitochondria were stained with MitoTracker and visualized by red fluorescence. The confocal images showed that in untreated A2058, SAN and BJ-5ta cells, SOD2 was exclusively present in the mitochondria, because only a merging yellow fluorescence was evident. However, a different picture emerged when these cells were incubated for 24 h with 150 μM diclofenac. In particular, in A2058 and SAN cells, besides its normal localization in mitochondria (yellow fluorescence), SOD2 was visualized also in the cytosol (green fluorescence). On the other hand, SOD2 was exclusively present in the mitochondria of BJ-5ta cells treated with the drug.

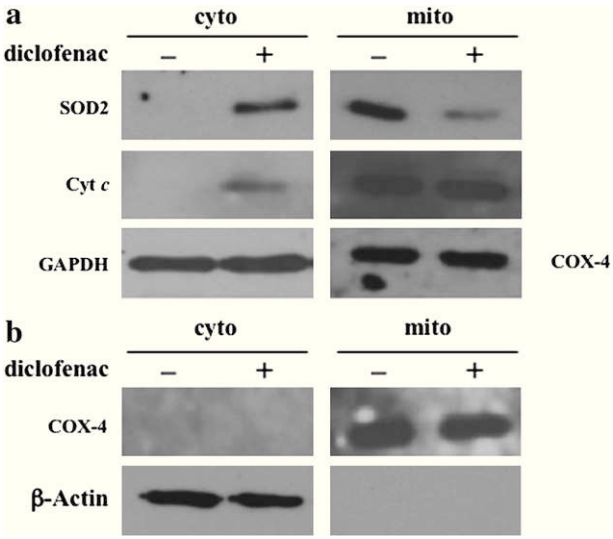

Fig. 9. Effect of diclofenac on SOD2 and cytochrome *c* subcellular localization in A2058. Cells were incubated for 24 h with vehicle alone or 150 μM diclofenac and then the cytosolic and mitochondrial protein extracts were prepared. (a) Western blotting analysis of the subcellular localization of cytochrome *c* and SOD2 was carried out, using GAPDH or COX-4 as loading control of the cytosolic or mitochondrial fraction, respectively. (b) Evaluation of the purity of subcellular fractions was monitored by Western blotting, using β-actin or COX-4 as specific cytosolic or mitochondrial marker, respectively.

It is known that caspase-9 activation is a typical event of the intrinsic pathway of apoptosis [32]. Therefore, we have measured the enzymatic activity of caspase-9 upon the diclofenac treatment. The data shown in Fig. 11 indicate that the activity of caspase-9 underwent a significant enhancement in both melanoma cell lines after 24-h incubation with 150 μM diclofenac. The higher levels of activity reached by caspase-9 in A2058 with respect to SAN cells reflect the higher initial level of this activity in untreated A2058 cells. In the same conditions, no increase of caspase-9 activity was measured in the nonmalignant BJ-5ta cells. It is worth mentioning that the responsiveness to the diclofenac treatment was higher in SAN compared to A2058, as emerging by the values of 4.3 and 2.2 measured as fold-induction over untreated cells for SAN and A2058, respectively. Therefore, when considering the different responsiveness of SAN and A2058 to diclofenac, the results obtained with caspase-9 are in the same direction of the previous data indicating an apoptosis induction, such as those obtained with propidium iodide and caspase-3 assays. Collectively, these results suggest that in melanoma cells diclofenac affects the mitochondrial functionality, by inducing the apoptosis through the intrinsic pathway.

4. Discussion

Melanoma is one of the most aggressive cutaneous cancers; moreover, in last years, its incidence is growing remarkably and, contrary to other neoplasms, a relatively younger population is becoming affected by this tumor. Apoptosis represents an efficient and physiological strategy through which the organism eliminates neoplastic cells; however, melanoma cells, both *in vivo* and *in vitro*, are quite refractory to apoptosis [33]. It is known that many chemotherapeutic agents induce cell death in cancer cells through an apoptotic mechanism. In the present work we have evaluated if diclofenac, an anti-inflammatory agent even used as a coadjutant in chemotherapy [34], may have cytotoxic effects in melanoma cells. In a previous paper we showed that diclofenac had a pro-apoptotic effect in the neuroblastoma cell line SH–SY5Y [13]; a similar action by diclofenac was reported in other cancer cell lines [4,7,11,35,36]. In this paper we show that diclofenac is cytotoxic for A2058 and SAN melanoma cells, whereas no significant effects were evident in the nonmalignant fibroblast BJ-5ta. In particular, we have demonstrated that, during the diclofenac treatment, the melanoma cells die through an apoptotic mechanism, a process associated to other events, such as increase of intracellular ROS levels, reduction of Bcl-2/Bax ratio, mitochondrial release of cytochrome *c*, increase of caspase-9 and -3 activity, reduction of SOD2 protein levels and cytosolic translocation of this mitochondrial enzyme. The usage of BJ-5ta as nonmalignant cell control, instead of a melanocyte cell line, was suggested by the consistent and ubiquitous presence of fibroblasts in any body tissue; therefore, in a therapeutic perspective, unspecific cell death should represent a more serious collateral effect in ubiquitous cell types than in rare and specialized cell types, such as melanocytes.

The morphological changes induced by diclofenac in A2058 and SAN suggest that this drug induces cellular alterations typical of cells undergoing apoptosis. This pro-apoptotic effect was selective on malignant melanoma cells, because no significant morphological alterations were evident in the fibroblast BJ-5ta. The increase of PI incorporation and caspase-3 activity observed in melanoma cells confirmed the susceptibility of A2058 and SAN to diclofenac.

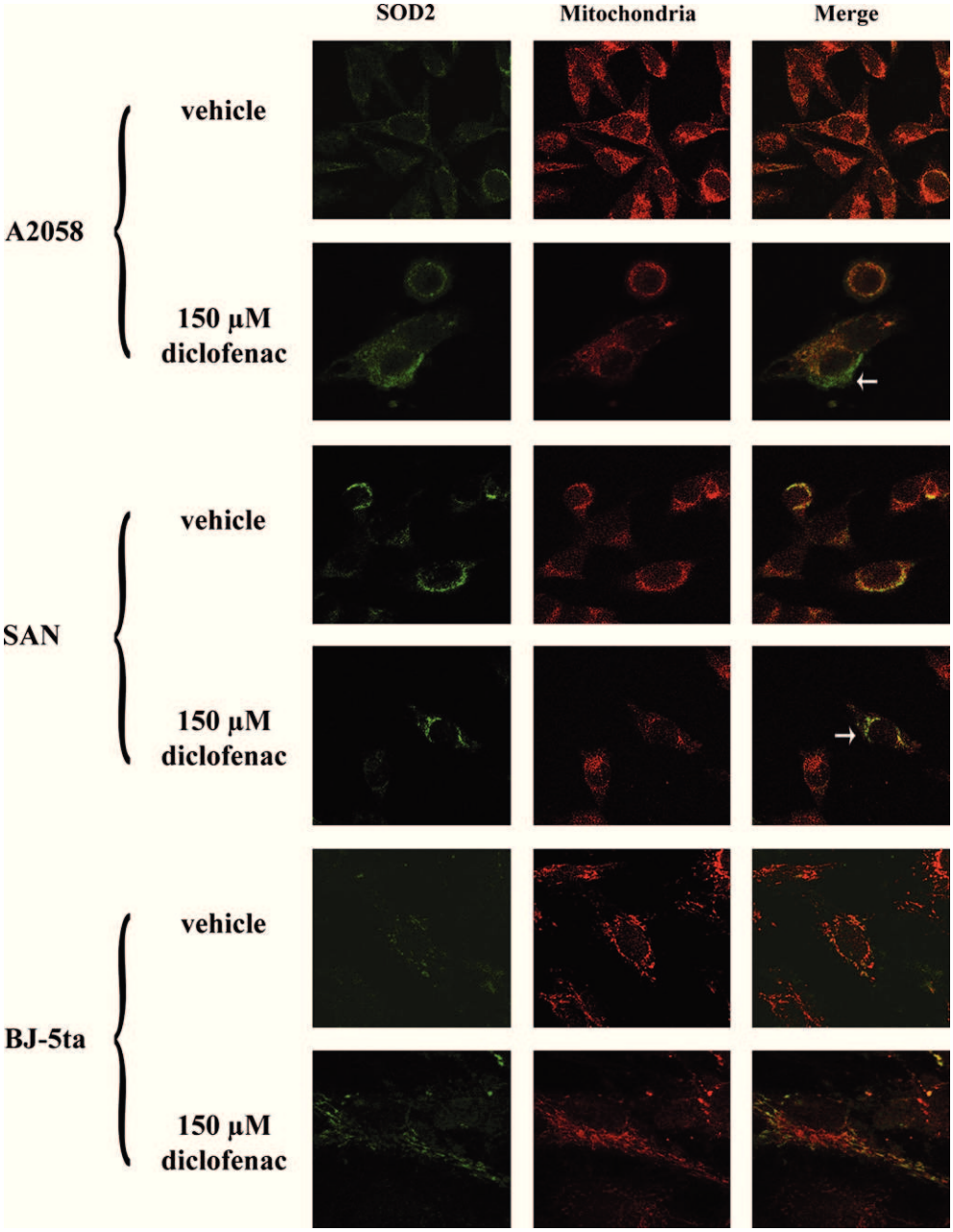

Fig. 10. Confocal images of SOD2 subcellular localization in melanoma and fibroblast cell lines upon diclofenac treatment. A2058, SAN and BJ-5ta cells were incubated for 24 h with vehicle alone or 150 μM diclofenac. Mitochondria were stained with 90 nM MitoTracker Red for 1 h (red fluorescence); then, the SOD2 immunofluorescent staining (green fluorescence) was performed using a polyclonal anti-SOD2 antibody, followed by FITC-conjugated antibody. The degree of spatial overlap in SOD2 distribution and MitoTracker Red staining is shown by yellow fluorescence, corresponding to a superimposition of red and green images (merge). Arrows indicate the presence of SOD2 in the cytosol.

To investigate on the mechanisms involved in the cellular effects induced by diclofenac, the expression profile of two members of Bcl-2 family, the anti-apoptotic Bcl-2 and the pro-apoptotic Bax was analyzed. It is known that Bcl-2 is actively involved in the development of chemo- and radio-resistance [24,37,38] and its up-regulation is associated to apoptosis resistance in many tumors, especially melanomas [24,39]. Bax plays a key role in apoptosis by acting on mitochondria permeability. In particular, this protein is retained away from mitochondria through its interaction with some anti-apoptotic Bcl-2 family proteins [26]. The decrease of Bcl-2/Bax ratio in A2058 and SAN cells is indicative of the involvement of these proteins in the pro-apoptotic effect of the drug on melanoma cell lines, even considering the slight increase of this ratio in BJ-5ta. In particular, our data suggest that diclofenac treatment, reducing the Bcl-2 protein levels in melanoma cell lines, affects the capacity of Bcl-2 to interact with Bax and to block it.

Compared to nonmalignant counterparts, cancer cells usually have higher intracellular ROS levels [40–42]. These highly reactive molecules regulate cellular proliferation and eventually cooperate to the oncogenic cell transformation [43]. On the other hand, ROS are also involved in the activation of natural defences against the appearance and propagation of neoplastic cells [44]. The observed increase of intracellular ROS detected in both melanoma cells likely contributes to the diclofenac-induced apoptosis. In particular, the early increase of superoxide anions, deriving from the activity of some enzyme systems involved in the detoxification of the drug

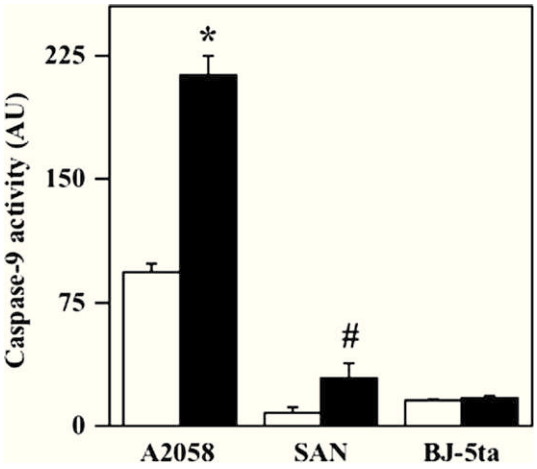

Fig. 11. Effect of diclofenac on caspase-9 activity in A2058, SAN and BJ-5ta. Cells were incubated for 24 h with vehicle alone (white bars) or 150 μM diclofenac (black bars); caspase-9 enzymatic activity was reported as Arbitrary Units (AU). Data from triplicate experiments are reported as mean ± SD. $^{\#}P < .05$ and $^{*}P < .01$ compared to untreated cells.

[18], could, in turn, trigger the formation of other ROS at a later stage. Hence, the increase of cellular ROS levels can affect the mitochondrial function. Indeed, the uncoupling among the enzyme systems involved in the mitochondrial electron chain transport likely takes place, because of the typically known sensitivity to ROS damage displayed by these macromolecular complexes. Because SOD2 represents the major antioxidant enzyme in the mitochondria, an efficient activity of this protein is required to counteract the mitochondrial dysfunction induced by oxidative stress. Furthermore, in case of prolonged high ROS levels, a programmed cell death becomes evident in various disease contexts [45]. Controversial issues appeared on SOD2 involvement in neoplastic cells. In some cases the expression level of SOD2 is high in aggressive human tumors, and an increase of its enzymatic activity is associated to the resistance of some tumors to chemo- and radiotherapies [46–49]. Under this concern, compounds causing a specific down-regulation of SOD2 in neoplastic cells are attractive molecules. In a previous work, we reported that diclofenac induces an increase of ROS concomitant to a decrease of SOD2 levels in the neuroblastoma cell line SH–SY5Y [13]. In this work, the decrease of both SOD2 protein levels and enzymatic activity in melanoma cells indicates that its reduction could represent another factor rendering these cells more vulnerable to the diclofenac-induced apoptosis respect to immortalized fibroblasts. In fact, an active energy metabolism produces high ROS levels, especially superoxide anions, in cancer cells; this constant oxidative stress condition renders the neoplastic cells more dependent on SOD2 enzyme and probably more susceptible to SOD2 modulators, respect to normal cells [50]. Therefore, these data suggest that SOD2 could represent a potential therapeutic target, because its inhibition contributes to the induction of cell death in cancer cells. Moreover, in some cases SOD2 exerts an anti-apoptotic role, demonstrated also by the resistance to radiation-induced damage reported for cell lines overexpressing SOD2 [51]. On the other hand, other issues attribute an anti-oncogenic role to SOD2. For instance, overexpression of SOD2 was associated to a regression of the neoplastic phenotype, as well as to a decrease of the metastatic potential [52–55]. These somehow controversial results are not surprising, because it is important to note that an intracellular redox imbalance could affect either the development or the regression of a malignant phenotype. All these observations promoted a further investigation on the effect exerted by SOD2 in our experimental system. Indeed, when SOD2 gene expression was silenced, the cytotoxic effect of diclofenac was amplified in both A2058 and SAN cells, thus pointing to the protective effect displayed by this antioxidant enzyme during drug treatment. This result is in agreement with others studies in which squamous cell carcinomas, transfected with an anti-sense SOD2 and then inoculated into mice, proliferated more slowly in the recipient mice; moreover, these latter also showed a longer life span compared to controls [56].

The appearance of SOD2 in the cytosolic fraction of both melanoma cells after the diclofenac treatment, represents another interesting and poorly documented issue of this work. However, the presence of SOD2 in the cytosol could be a cause or a consequence of the mitochondrial dysfunction induced by the drug. Because SOD2 is enzymatically active only in the mitochondrial compartment [57], the presence of an inactive SOD2 in the cytosol could affect the cellular redox balance and then amplify the pro-apoptotic effect of diclofenac. In fact, it was reported that sodium selenite or bile salts provoke the cytoplasmic SOD2 localization, thus contributing to the mitochondrial dysfunction [58].

Two different apoptotic pathways were essentially described: an extrinsic pathway, promoted by death receptors, and an intrinsic one, mediated by mitochondria. This organelle exerts a central role in the control of the apoptotic process, mainly when cell death is induced by drugs [59]. In fact, Bouchier-Hayes and colleagues [60] assume that mitochondria can be considered as targets for pharmacological intervention. Our data indicate that the observed pro-apoptotic effect of diclofenac in melanoma cells is mediated through the intrinsic mitochondrial pathway. Indeed, during the diclofenac-induced apoptosis, a reduction of the Bcl-2/Bax ratio and a translocation of both cytochrome *c* and SOD2 from mitochondria to cytosol take place. Moreover, these events are associated to a significant increase of caspase-9 activity in treated cells, whose activation is notoriously mediated by the cytosolic translocation of cytochrome *c* [32].

In conclusion, our data suggest that diclofenac exerts its cytotoxic effect targeting the mitochondria. In particular, the increase of ROS levels, modulating the Bcl-2/Bax ratio [61], can, in turn, provoke the cytosolic translocation of cytochrome *c* and then caspase-9 activation. Furthermore, the reduction of SOD2 levels and its appearance in the cytosol, together with the effects described for the other parameters investigated, can be considered as signals of the mitochondrial dysfunction induced by diclofenac in the melanoma cell lines. On the other hand, no significant alteration of all these parameters was revealed in the nonmalignant fibroblast cell line BJ-5ta, thus showing its lower sensitivity to the pro-apoptotic effects of diclofenac. To this aim, it has been reported that in some fibroblast cell lines, such as a human dermal fibroblast [62] or the mouse fibroblast 3T3 [63], lower levels were reported for the enzymes systems involved in drug metabolization, a feature eventually explaining the different resistance either among fibroblast and melanoma cells or among various fibroblast lines.

Recently, many studies identified mitochondria as central players in the process of neoplastic cellular transformation [42,64]. In fact, these organelles are more numerous in cancer cells and this circumstance leads to an increase of ROS levels; as a consequence, a number of DNA mutations takes place, thus contributing to exacerbate cell malignancy and to escape from senescence. Hence, it is interesting to identify new drugs possessing anti-neoplastic activity; in particular, these molecules should trigger the mitochondrion to produce further ROS, so to induce the selective death of cancer cells. These drugs, associated to conventional treatments, may amplify the therapeutic properties and circumvent a possible chemoresistance; furthermore, these novel molecules could represent an alternative therapeutic strategy, when used in combination

with inhibitors of proteins that modulate negatively apoptosis, such as SOD2.

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ANALYSIS OF EXTRACELLULAR SUPEROXIDE DISMUTASE IN FIBROBLASTS FROM PATIENTS WITH SYSTEMIC SCLEROSIS

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Systemic sclerosis (SSc) is a chronic disease of connective tissue characterized by vascular damage, autoantibody production and extensive fibrosis of skin, skeletal muscles, vessels and visceral organs. Fibrosis is a biological process involving inflammatory response and reactive oxygen species (ROS) accumulation leading to fibroblast activation. Extracellular superoxide dismutase (SOD3), a copper and zinc superoxide dismutase, which is expressed in selected tissues, is secreted into the extracellular space and catalyzes the dismutation of superoxide radical to hydrogen peroxide and molecular oxygen. Moreover, SOD3 is associated to inflammatory responses in some experimental models. In this paper we analysed, by RT-PCR and immunofluorescence, SOD3 expression and intracellular localization in dermal fibroblasts from both healthy donors and patients affected by diffuse form of SSc. Moreover, we determined SOD3 enzymatic activity in fibroblast culture medium with the xanthine/xanthine oxidase method. Increased expression of SOD3 mRNA was detected in systemic sclerosis fibroblasts (SScF), as compared to control healthy fibroblasts (HF), and SOD3 immunofluorescence staining displayed a characteristic pattern of secretory proteins in both HF and SScF. Superoxide dismutase assay demonstrated that SOD3 enzymatic activity in SScF culture medium is four times more than in HF culture medium. These data suggest that an alteration in SOD3 expression and activity could be associated to SSc fibrosis.

Systemic sclerosis (SSc) is a multisystemic disease characterized by inflammation, vascular injury, circulating autoantibodies and extensive fibrosis of vessels, skin, skeletal muscles, and visceral organs, that overthrow the architecture of the affected organ (1-2). Fibrosis, due to increased synthesis of extracellular matrix proteins by activated fibroblasts, is a biological process associated to an inflammatory response and modulated by many cytokines (3). Furthermore, several studies demonstrated a role of oxidative stress in SSc pathogenesis. In fact, in patients with SSc, decreased serum levels of antioxidants or increased levels of oxidative damage markers have been demonstrated (1). Moreover,

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reactive oxygen species (ROS) accumulation is implicated in fibroblast activation through different mechanisms: changes in the protease-antiprotease balance (4), autoantibody production (1), positive interaction with TGF-β and PDGF, in the signalling leading to fibrosis, and constitutive activation of pathway linking Ras with ERK1/2 and ROS (2, 5-6).

The extracellular superoxide dismutase (SOD3) is copper and zinc superoxide dismutase that removes superoxide anions from the extracellular microenvironment, catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and molecular oxygen (7). SOD3 is expressed in vascular smooth muscle cells, in alveolar type II cells, in glial cells, in several human fibroblast lines and is secreted into the extracellular space where it regulates extracellular matrix homeostasis (7). Moreover, SOD3 is associated to inflammatory responses in several experimental models (8-9).

In this paper we investigated the expression and intracellular localization of SOD3 in dermal fibroblasts from both healthy donors and SSc patients, and evaluated SOD3 enzymatic activity in fibroblasts culture medium.

MATERIALS AND METHODS

Patients

Four females (mean age 38.5±4.6 years), affected by diffuse form of systemic sclerosis (mean duration of disease 4.3±2.5 years), were enrolled in our study after analysis in the Rheumatology Surgery Center of Clinical and Experimental Medicine of Federico II Medical School of Naples. Two of them presented esophagopathy, only one was affected by lung fibrosis; none of them was treated with penicillamine or steroids. Fine needle punch biopsies from the affected skin area (forearm) were carried out. Surgical fragments from the same sites were obtained from 2 volunteers (females, 37±1 years old) who underwent aesthetic surgery and were considered as normal controls. Each fragment was mechanically dissociated under the stereo microscope, for *in vitro* culture. The investigation conforms to the principles outlined in the Declaration of Helsinki and informed consent was obtained from all patients.

Cell culture

Healthy fibroblasts (HF) and systemic sclerosis fibroblasts (SScF) from skin biopsies were obtained as follows: surgical fragments were mechanically dissociated under a light microscope and then submitted to tripsynization for 30 minutes at 37°C. After repeated PBS washing, microfragments were plated and cultured in Dulbecco's minimal essential medium (DMEM) (BIOWhittaker, Belgium), containing 10% fetal calf serum (GIBCO, Grand Island, NY, USA), 200 mM L-glutamine, penicillin (100 mg/ml) and streptomycin (100 mg/ml). The plates were incubated at 37°C, in the presence of 5% CO_2 and the medium was removed every 3 days. The outgrowth of fibroblasts from the bioptic fragments was observed after 2-4 weeks. Once the fibroblasts were 75% confluent, they were detached with 0.25% trypsin-EDTA and plated. All experiments were performed only from early-passage fibroblasts (< 5) to avoid the possibility of phenotypic changes during extended subculture.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells using Trizol solution (GIBCO BRL, Life Technologies, Rockville, Md, USA). After analysis of yield and integrity of each RNA sample, 5 µg of total RNA were reverse transcribed by using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Arlington Heigths, IL, USA). Two µl of products of each samples were used for subsequent PCR amplification with primers designed for both SOD3 and the GAPDH housekeeping genes, using the VWR Taq DNA Polymerase (VWR International, Belgium) according to the manufacturer's instructions. Samples without cDNA were used as negative controls for PCR reactions. The PCR reactions were performed with 25 or 30 cycles using the following conditions: an initial denaturation step for 5 min at 95°C; denaturation for 30 sec at 94°C, 1 min of annealing for SOD3 and GAPDH, at 61°C and 58°C respectively, extension for 30 sec at 72°C; final extension step for 2 min at 72°C. The primers used for PCR and the expected size of their amplification products were:

a) SOD3 forward primer CCTGCGTTCCTGGGCTGGCTG, SOD3 reverse primer CTCCGTGACCTTGGCGTACAT, 219 bp;

b) GAPDH forward primer CACCATCTTCCAGGAGCGAG, GAPDH reverse primer TCACGCCACAGTTTCCCGGA, 372 bp.

The specific primers for SOD3 were designed with Oligo4 software. GAPDH primers were gently gifted by Prof. P. Arcari. The amplified products were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of SOD3 cDNAs were estimated by densitometric scanning and normalized against GAPDH loading controls. Densitometric analyses were

performed with Image J 1.429 software.

Immunofluorescence

Hydrogen peroxide cell treatment was performed as previously described (10). Cells plated on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked in donkey serum, diluted 1:10 in PBS 1X, for 60 min at room temperature. Coverslips were incubated with a primary antibody mouse monoclonal anti-SOD3 (Abcam plc, Cambridge Science Park, Cambridge, UK), or with non-immune mouse serum, diluted 1:50 in PBS 1X, for 1 hour at 37°C, and subsequently incubated with a FITC donkey anti-mouse secondary antibody (Jackson ImmunoResearch Europe Ltd. Unit 7, Acorn Business Centre, Oaks Drive, Newmarket, Suffolk, UK CB8 7SY) diluted 1:50 in PBS 1X, for 1 hour at 37°C. The cell nuclei were labelled with DAPI. Slide mounting was done in Vectashield. Signals were visualized with a Leica DMLB fluorescent microscope. Images from SOD3 and DAPI cell fluorescence patterns were processed as one-color images, merged and reconstructed using Adobe Photoshop software.

SOD assay

To evaluate SOD3 enzymatic activity, fibroblast culture medium was previously concentrate using centricon™ 30 microconcentrator (Amicon Corporation Scientific Systems Division, Danvers, MA). SOD3 activity was measured in 100 mM potassium phosphate pH 7.8, 0.1 mM Na_2EDTA at 25°C by the inhibition of the cytochrome *c* reduction, caused by superoxide anions generated with the xanthine/xanthine oxidase system, and determined by spectrophotometric method (11). One unit of SOD3 activity was defined as the amount of enzyme that caused 50% inhibition of cytochrome *c* reduction. The SOD3 enzymatic assay was conducted both in absence and presence of 2 mM KCN, a specific inhibitor of Cu/Zn SOD enzymes (12). Protein concentration was determined by the Bradford method, using bovine serum albumin as standard (13).

RESULTS

Analysis of SOD3 mRNA expression level

We performed RT-PCR on total RNA to investigate SOD3 mRNA expression in dermal fibroblasts from both healthy donors and SSc patients.

RT-PCR analysis showed clear differences in the expression level of SOD3 mRNA (Fig. 1A) that is approximately three times higher in SScF than in HF (Fig. 1B). Moreover, RT-PCR analysis, performed with different dilutions of cDNA templates (Fig. 1C) and with different number of PCR cycles (Fig. 1D), yields different ethidium bromide fluorescence signals and shows that the amplification reactions are in a linear range.

SOD3 intracellular localization

To investigate the intracellular localization of endogenous SOD3, both HF and SScF were immunostained with a monoclonal anti-SOD3 antibody, followed by a donkey FITC anti-mouse antibody (Fig. 2 A and D). Then nuclei were labelled with DAPI (Fig. 2 B and E). Furthermore, as negative control, the fibroblasts were immunostained with non-immune mouse serum, followed by donkey FITC anti-mouse antibody (Fig. 2G) and then nuclei were labelled with DAPI (Fig. 2H). SOD3 immunofluorescence staining displayed a characteristic pattern of secretory proteins in both HF and SScF, and did not show nuclear localization (Fig. 2 A, C, D and F). Furthermore, SOD3 immunostaining is more intense in SScF (Fig. 2D) than in HF (Fig. 2A). To evaluate the effects of oxidative stress on the intracellular localization of endogenous SOD3, both HF and SScF were treated with 5 mM and 50 mM H_2O_2: also in these conditions no nuclear localization was observed (not shown).

Analysis of SOD3 enzymatic activity

To determine SOD3 enzymatic activity, culture medium from both HF and SScF was concentrated and SOD assay was performed using the xanthine/xanthine oxidase method. As shown in Fig. 3, SOD3 enzymatic activity of culture medium from SScF is four times higher than that determined in culture medium from HF. Moreover, it is important to note that SOD3 activity was completely inhibited in the presence of 2 mM KCN and that no superoxide dismutase activity was detected in Dulbecco's complete medium (not shown).

DISCUSSION

Systemic sclerosis (scleroderma) is a rare and complex disease that is characterized by a prevalence ranging from 50 to 300 cases per 1 million persons and an incidence ranging from 2.3 to 22.8 cases per 1 million persons per year (2). Hence, it is important to consider that the number of patients in our study

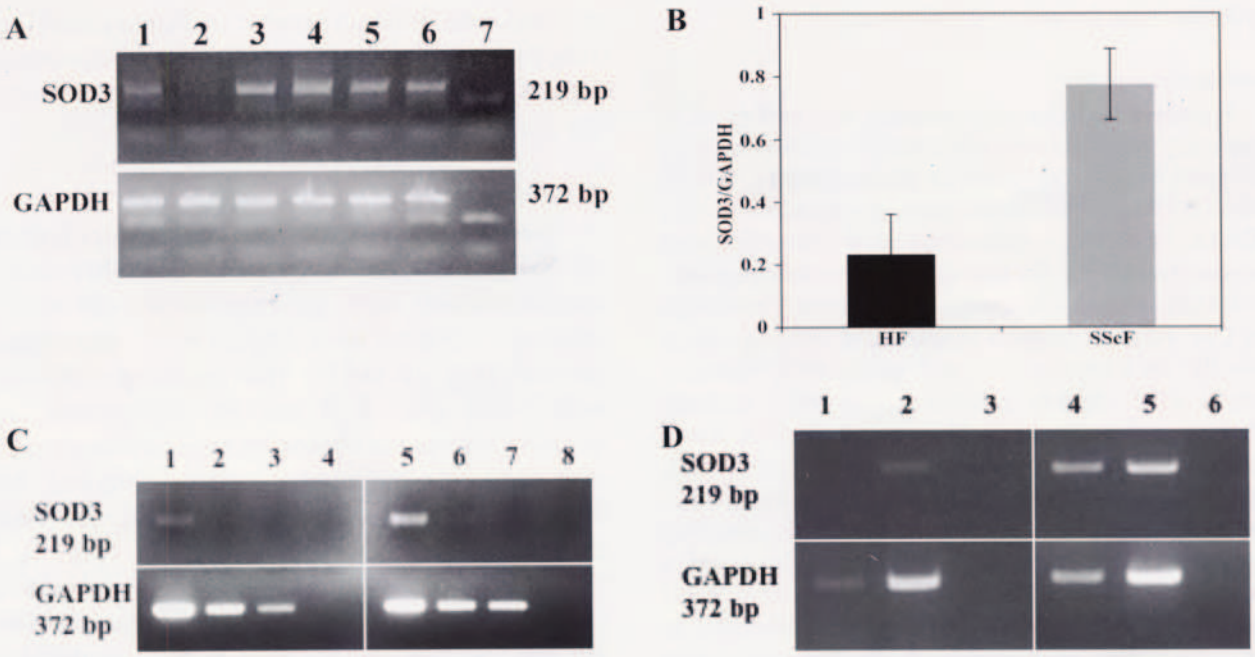

Fig. 1. *Analysis of SOD3 mRNA expression in both HF and SScF by RT-PCR.* ***A)*** *The gel shown in figure is representative of three RT-PCR experiments. GAPDH was used as loading control in each PCR reaction. Lanes 1, 2, cDNAs from HF; lanes 3, 4, 5, 6, cDNAs from SScF; lane 7, negative control. The PCR reactions were performed using cDNAs not diluted and with 25 cycles.* ***B)*** *Densitometric analysis of SOD3 expression. The data represent the means of densitometric ratio between SOD3 and GAPDH cDNAs of three RT-PCR experiments ±S.D.* ***C)*** *RT-PCR reactions performed with different dilutions of the cDNAs templates. Lanes 1, 2, 3 cDNAs from HF not diluted, 1:10 diluted and 1:100 diluted, respectively. Lanes 5, 6, 7, cDNAs from SScF not diluted, 1:10 diluted and 1:100 diluted, respectively. Lanes 4, 8, negative controls. The PCR reactions were performed with 25 cycles.* ***D)*** *RT-PCR reactions performed with different numbers of PCR cycles. Lanes 1, 2, PCR of cDNAs, from HF, performed respectively with 25 and 30 cycles. Lanes 4, 5, PCR of cDNAs, from SScF, performed respectively with 25 and 30 cycles. Lanes 3 and 6, negative controls. The gels shown in figures are representative of three independent RT-PCR experiments, performed on total RNA from dermal fibroblasts of two healthy donors and four SSc patients.*

is only apparently small. There are two types of SSc: limited cutaneous scleroderma, characterized by fibrosis restricted to the hands, arms, face and diffuse cutaneous scleroderma that affects a large area of the skin and compromises one or more internal organs (2).

In this paper we report that both SOD3 gene expression and extracellular enzymatic activity are higher in dermal fibroblasts from patients affected by diffuse form of SSc than in fibroblasts from healthy donors.

It is well-known that sclerodermic fibroblasts exhibit increased production of extracellular matrix proteins and are able to increase cell adhesion plaques accompanied by an increased expression of granulocyte colony stimulating factor receptor (2, 14). The phenotype of sclerodermic fibroblasts persists *in vitro* without exogenous stimuli and is associated with the development of several autocrine cytokine/chemokine loops (15), and with increased Sp1 transcription factor phosphorylation that is involved in the COLIA2 gene upregulation (3). Furthermore, TGF-β signalling is the most important factor in the autocrine cytokine/chemokine loops of sclerodermic fibroblasts (3, 15-17).

It has been shown that SOD3 expression in

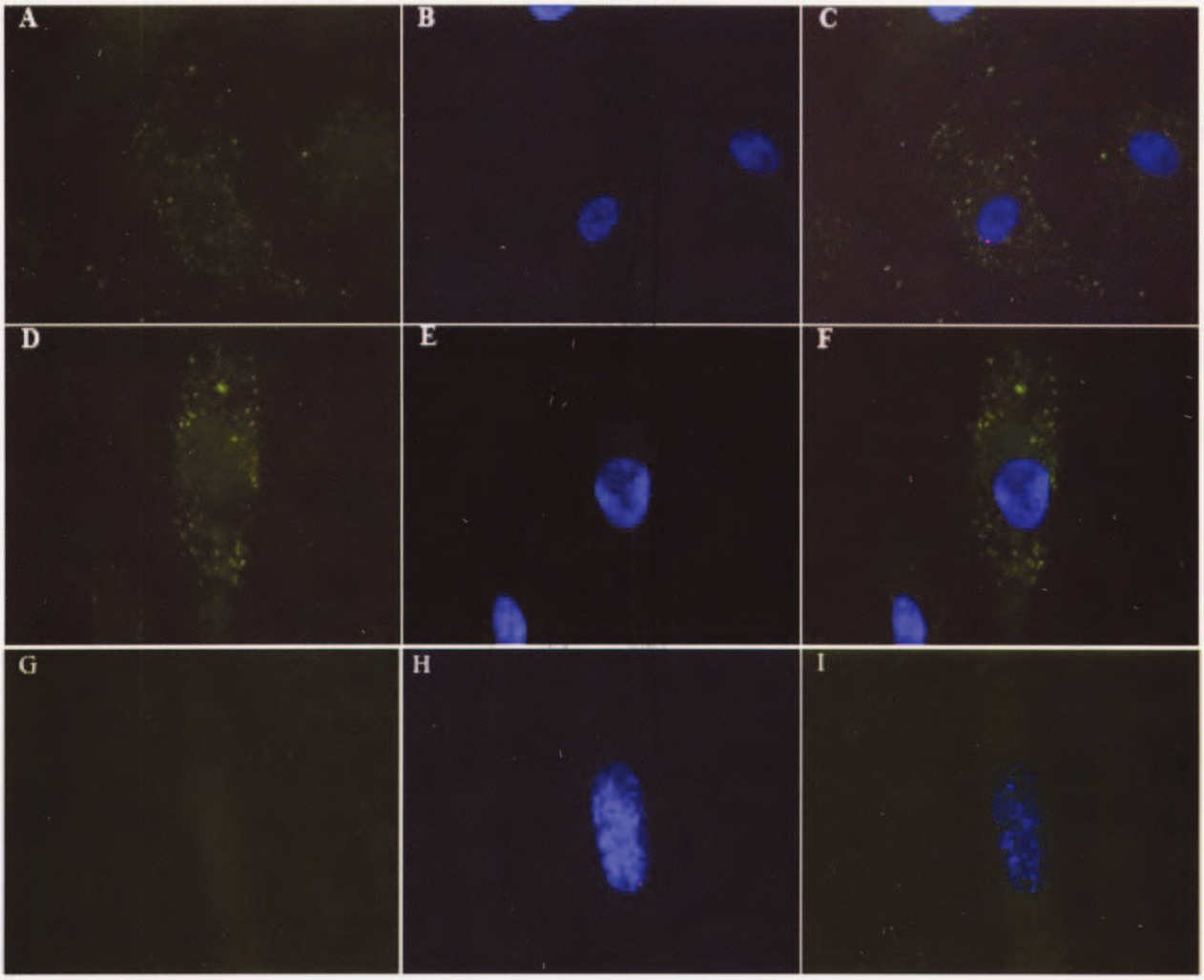

Fig. 2. *SOD3 intracellular localization. Co-staining of SOD3 with DAPI in HF (A-C) and SScF (D-F). Cells were stained with a monoclonal anti-SOD3 antibody followed by a donkey FITC anti-mouse antibody (**A**, **D**). The same cells have been stained with DAPI (**B**, **E**) that labels nuclei. The degree of spatial overlap in the SOD3 and DAPI distribution is shown by overlay pictures (**C**, **F**). Co-staining of the fibroblasts with non-immune mouse serum and DAPI (**G-I**). The immunofluorescences shown in figure are representative of six independent experiments performed on dermal fibroblasts from two healthy donors and four SSc patients. Magnification X100.*

human dermal fibroblasts is not responsive to oxidative stress (18) but is regulated by various inflammatory cytokines and that in lung fibroblasts Sp1 transcription factor interacts with human SOD3 promoter and stimulates SOD3 promoter activity (19-20). Hence, it is possible that SOD3 gene expression regulation in SScF is connected to autocrine loops involving TGF-β, PDFG, IL1 and other cytokines, whose role in fibroblast activations has not yet been clarified. However, the lack of knowledge about the role of other cytokines, involved in autocrine loops, explains why the treatment with a monoclonal anti-TGF-β1 antibody was ineffective in a clinical trial (15).

Other factors, with profibrotic activity, that could regulate SOD3 expression in SScF, are represented by the vasoactive peptides, endothelin-1 and angiotensin II. Serum endothelin-1 levels are increased in SSc patients, and can promote myofibroblast differentiation, induce the expression

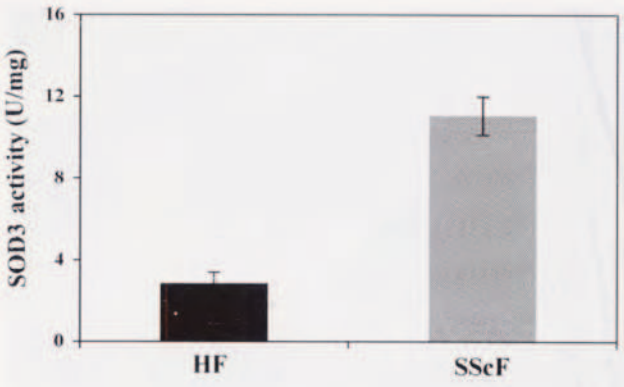

Fig. 3. *Measurement of SOD3 activity in fibroblasts culture medium. SOD3 enzymatic activity was determined both in HF and SScF culture medium as described in Materials and Methods. The results represent the means of three independent experiments ± S.D.*

of collagen type I and III, and inhibit MMP-1 expression and activity (3). Angiotensin II serum levels, in patients with diffuse cutaneous SSc, were significantly higher than those in healthy donors, and angiotensin II mRNA was expressed in dermal fibroblasts from SSc patients but not in normal fibroblasts (21). Furthermore, in vascular smooth muscle cells, both SOD3 expression and secretion were upregulated by angiotensin II and endothelin-1 (22).

Here we show that SOD3 enzymatic activity in SScF culture medium is four times more than in HF culture medium. Previous studies have shown that the rate of SOD3 degradation in culture medium is low (23); therefore the difference of SOD3 activity, observed between HF and SScF culture medium, should coincide with SOD3 amount in culture medium and could also be associated to differences in both synthesis and secretion rate between HF and SScF. Hence, the increase of both SOD3 gene expression and secretion observed in SScF could have several physiological consequences on SSc fibrosis. First of all, because SOD3 binds to type I collagen and protects this structural protein from oxidative fragmentation (24), the high amounts of SOD3, synthesized and secreted by SScF, bind to collagen type I and could reduce the collagen turnover and contribute to SSc fibrotic process.

Moreover, also SOD3 enzymatic activity could affect SSc fibrotic process. In fact, hydrogen peroxide increases cytokines secretion by Th2 cells and promoted Th2 predominant response associated to SSc fibrosis (25, 1); it can traverse lipid membrane and induce proliferation and upregulation of extracellular matrix proteins of normal fibroblasts (2, 1, 26).

This work also analysed the intracellular localization of endogenous SOD3. Immunostaining of SOD3 displayed a characteristic pattern of secretory proteins in both HF and SScF, but nuclear localization was not observed, also in fibroblasts treated with hydrogen peroxide. Furthermore, it is interesting to note that SOD3 immunostaining is more intense in SScF than in HF and this is in agreement with SOD3 gene expression and activity results. In human fibroblast cell lines, which express SOD3 and secrete the enzyme in the medium, a fraction of SOD3 remains inside the cell for a long time, but its intracellular localization has not been characterized (23). Previous studies have been demonstrated that SOD3 can localize in the nucleus where it could have a biological role (27-29). In fact, *in vitro* experiments have shown that exogenous SOD3 can be translocated from extracellular space into cytosol and nucleus of a mouse preadipocyte cell line and that nuclear translocation was enhanced by hydrogen peroxide treatment (27, 10). Our immunofluorescence experiments suggest that the biological role of endogenous SOD3 does not lie in the nucleus of either HF or SScF.

The results presented in this paper suggest that the alteration in both expression and secretion levels of SOD3 could affect SSc fibrosis and may open new perspectives for the antioxidant genes study. In fact, to understand the biological role of antioxidant genes in diseases such as SSc there is need to study the cellular and extracellular environment in which the products of these genes work. However, we will perform further studies to identify the factors and the pathway regulating SOD3 expression and secretion in SScF, to suggest new SSc therapeutic perspectives.

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Research Article

Diclofenac-Induced Apoptosis in the Neuroblastoma Cell Line SH-SY5Y: Possible Involvement of the Mitochondrial Superoxide Dismutase

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Diclofenac, a nonsteroidal anti-inflammatory drug, induces apoptosis on the neuroblastoma cell line SH-SY5Y through a mitochondrial dysfunction, affecting some antioxidant mechanisms. Indeed, the time- and dose-dependent increase of apoptosis is associated to an early enhancement of the reactive oxygen species (ROS). Mitochondrial superoxide dismutase (SOD2) plays a crucial role in the defence against ROS, thus protecting against several apoptotic stimuli. Diclofenac decreased the protein levels and the enzymatic activity of SOD2, without any significant impairment of the corresponding mRNA levels in the SH-SY5Y extracts. When cells were incubated with an archaeal exogenous thioredoxin, an attenuation of the diclofenac-induced apoptosis was observed, together with an increase of SOD2 protein levels. Furthermore, diclofenac impaired the mitochondrial membrane potential, leading to a release of cytochrome *c*. These data suggest that mitochondria are involved in the diclofenac-induced apoptosis of SH-SY5Y cells and point to a possible role of SOD2 in this process.

1. Introduction

Reactive oxygen species (ROS), normally produced during the aerobic metabolism, function as second messengers involved in many cellular functions. On the other hand, when ROS level increases because of oxidant treatments and/or defective antioxidant systems, these highly reactive compounds and radicals become dangerous toxic agents. In fact, ROS may cause severe damages to several cellular macromolecules, including proteins, lipids, and DNA, thus contributing to the development of many pathological conditions. Indeed several evidences have been reported, indicating that the redox homeostasis is a finely regulated mechanism involved in normal cellular functions and prevention of several stress-associated pathologies [1].

Many drugs have toxic side effects, because they provoke an imbalance of the intracellular ROS level. In the past, cellular death due to a chemical injury was frequently linked to a necrotic process; now, it is clear that the main effect provoked by several drugs is the programmed cell death [2]. Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID) widely used in clinical therapeutics, has cytotoxic effects and induces apoptosis in many cultured cell lines [3, 4]. This behaviour is common even to other NSAIDs and some anticancer agents. Indeed, many experimental, epidemiologic and clinical studies suggest that NSAIDs, and in particular the highly selective cyclooxigenase-2 inhibitors, could act as anticancer agents [5, 6]. It has been reported that a combination of a specific NSAID and certain anticancer drugs has potential clinical applications. For instance,

diclofenac potentiates the chemotherapeutic effects of some drugs in neuroblastoma cell lines [7]. However, little is known about the effect of this NSAID on nervous cell lines [8], because most of the studies on this compound regard hepatic, gastric, or kidney cells [3, 4, 9]. In particular, ROS are involved in the diclofenac-induced apoptosis of cultured gastric cells as well as in nephrotoxicity in vivo [9, 10]; furthermore, an oxidative injury causes the mitochondrial permeability transition in diclofenac-treated hepatocytes [11]. However, the molecular mechanisms underlying the induction of apoptosis by diclofenac have not been clarified in neuronal cells.

In this paper, we have investigated the involvement of mitochondrial dysfunction in the mechanism of diclofenac-induced apoptosis in the neuroblastoma cell line SH-SY5Y. In particular, we have analyzed the role of the manganese superoxide dismutase (SOD2) in this process. SOD2 is a key enzyme of the mitochondrial matrix involved in the protection against oxidative stress, which converts the toxic superoxide anions to hydrogen peroxide and molecular oxygen. Numerous reports have demonstrated that SOD2 has an essential role in the protection against many apoptotic stimuli [12–14]. In fact, in mice a partial deficiency of the SOD2 gene (*sod2*(+/−)) increases the sensitivity to apoptosis [15], whereas its overexpression has an antiapoptotic effect. In particular, SOD2 is involved in the inhibition of the mitochondrial permeability transition after cell treatment with tumor necrosis factor-α or ionizing radiations [16–18], and blocks the Fas-mediated apoptosis [19, 20]. Our data show that the treatment of the neuroblastoma cell line SH-SY5Y with diclofenac induces a decrease in SOD2 protein level and an increase of the ROS concentration. This impaired redox balance predisposes the cell to apoptosis through a mechanism involving the mitochondrial pathway.

2. Materials and Methods

2.1. Chemicals. RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin G, streptomycin and trypsin were purchased from Cambrex. Diclofenac was obtained from Calbiochem. Rhodamine 123 (R123), dichlorofluorescein diacetate (DCFH-DA), and propidium iodide were purchased from Sigma. A protease inhibitor cocktail was obtained from Roche Diagnostics. Polyclonal antibody against human SOD2 was purchased from Upstate; polyclonal antibody against GAPDH was obtained from Cell signaling; polyclonal antibody against β-tubulin, goat polyclonal antibody against Cox-4, monoclonal antibody against cytochrome *c,* and each secondary antibody conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology. Recombinant thioredoxin A2 from the hyperthermophilic archaeon *Sulfolobus solfataricus* (*Ss*Trx) was obtained as previously reported [21]. All other chemicals were of analytical grade and were purchased from Sigma.

2.2. Cell Culture. The human neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection. SH-SY5Y cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin, in humidified incubator at 37°C under 5% CO_2 atmosphere. They were split and seeded in plates (75 cm^2) every three days and used for assays during exponential phase of growth.

2.3. Cytotoxicity Assay. An increasing concentration of diclofenac was added to cultures, and cells were incubated for different times. Cytotoxicity was quantitatively assessed by measurements of lactate dehydrogenase (LDH) activity released in the extracellular fluid from damaged or destroyed cells [22]. Briefly, different aliquots of cell incubation media were added to a 1-ml reaction mixture containing 0.1 M Tris-HCl, pH 7.5, 125 μM NADH, and incubated for 15 minutes at 30°C. The reaction started with the addition of 600 μM sodium pyruvate and was followed by the decrease in absorbance at 340 nm. The results were normalized to 100% death caused by cell sonication.

2.4. Evaluation of Apoptosis. To determine the number of apoptotic nuclei in diclofenac-treated cells, 3×10^4 cells/well were seeded into 96-well plates; at the end of each treatment, cell suspensions were centrifuged and pellets were resuspended in a hypotonic lysis solution containing 50 μg/ml propidium iodide. After incubation at 4°C for 30 minutes, cells were analysed by flow cytometry to evaluate the presence of nuclei with a DNA content lower than the diploid [23].

2.5. Detection of Intracellular ROS Content. The intracellular ROS level was detected using the oxidation-sensitive fluorescence probe DCFH-DA [11]. Briefly, cells were seeded into 6-well plate (3×10^5 cells/well) and treated with 150 μM diclofenac for different times. DCFH-DA was added in the dark at 10 μM final concentration 30 minutes before the end of each incubation; then cells were collected, washed in 10 mM sodium phosphate, pH 7.2 buffer containing 150 mM NaCl (PBS), and finally resuspended in 500 μL of PBS for the fluorimetric analysis. The measurement of the ROS levels was realised with a Cary Eclipse fluorescence spectrophotometer (Varian). Excitation and emission wavelengths were 485 nm and 530 nm, respectively; both excitation and emission slits were set at 10 nm.

2.6. Reverse Transcriptase Polymerase Chain Reaction. For the analysis of SOD2 mRNA expression, total RNA was extracted from 1×10^6 cells using a Trizol Reagent (Invitrogen) as described by the manufacturer. The yield and integrity of each RNA sample were checked spectrophotometrically at 260 nm and by agarose gel electrophoresis, respectively. Equal amounts of RNA (2–4 μg) were subjected to a reverse transcriptase polymerase chain reaction (RT-PCR), using a specific kit (Invitrogen) and random primers; three dilutions of cDNA were amplified by PCR using *Taq* DNA polymerase (Invitrogen). For DNA amplification of both SOD2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the PCR program was 5 minutes initial denaturation at 95°C, 20–23 cycles of amplification (95°C, 1 minute; 50°C, 1 minute; 72°C, 1 minute), final extension

step at 72°C for 10 minutes. The following primers were used: 5′d-TACGTGAACAACCTGAACGT-3′ (sense) and 5′d-CAAGCCATGTATCTTTCAGTTA (antisense) for SOD2; 5′d-CACCATCTTCCAGGAGCGAG-3′ (sense) and 5′d-TCACGCCACAGTTTCCCGGA-3′ (antisense) for GAPDH. The amplified cDNA products were analysed by agarose gel electrophoresis. SOD2 PCR products were normalized to the respective intensity of the house-keeping gene GAPDH.

2.7. Evaluation of Mitochondrial Membrane Potential. Mitochondrial membrane potential was evaluated by measuring the incorporation of the fluorescent probe R123. Briefly, cells were seeded into 6-well plate (3×10^5 cells/well), incubated at 37°C for 1 hour in the presence of 5 μM R123, washed twice with PBS and placed in fresh complete medium containing 150 μM diclofenac. After different times of drug treatment, the medium was withdrawn, and collected cells were washed twice with PBS. After detachment with trypsin, cells were harvested in PBS and centrifuged at 4°C for 10 minutes. Following aspiration of supernatant, the cellular pellet was resuspended in 500 μL of PBS. The fluorescence of cell-associated R123 was measured in the above-mentioned fluorescence spectrophotometer, using excitation and emission wavelengths of 490 nm and 520 nm, respectively; both excitation and emission slits were set at 10 nm. The fluorescence intensities were normalized versus the cell number.

2.8. Total Cell Lysates and Western Blotting Analysis. SH-SY5Y cells were plated at a density of 3×10^5 cells/well in 6-well plates, and 150 μM diclofenac was added to the cultures. After the drug treatment, cells were harvested, washed with PBS, and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycolate, 1 mM Na_3VO_4, and 1 mM NaF), supplemented with protease inhibitors and incubated for 30 minutes on ice. The supernatant obtained after centrifugation at 12,000× g for 30 minutes at 4°C constituted the total protein extract. Protein concentration was determined by the method of Bradford [24], using BSA as standard. Western blotting analysis was realised on equal amounts of each total protein extract. Briefly, protein samples were dissolved in SDS/reducing loading buffer, run on a 14% SDS-PAGE, then transferred to Immobilon P membrane (Millipore). The filter was incubated with the specific primary antibody at 4°C overnight and with the horseradish peroxidase-linked secondary antibody at room temperature for 1 hour. Membranes were then analyzed by an enhanced chemiluminescence reaction, using Super Signal West Pico kit (Pierce) according to the manufacturer's instructions; signals were visualized by autoradiography.

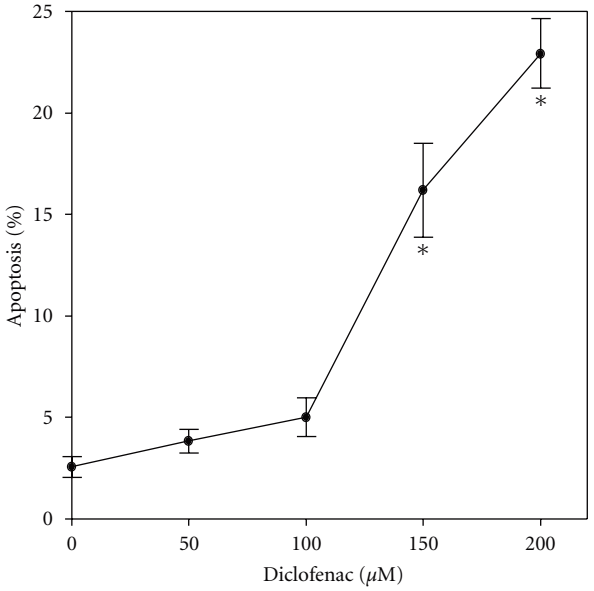
2.9. Subcellular Fractionation for Western Blotting Analysis of Cytochrome c. SH-SY5Y cells were plated at a density of 2×10^6 cells/plate (75 cm^2). After treatment with 150 μM diclofenac, cells were harvested, washed in PBS, then resuspended in buffer M (5 mM Hepes, pH 7.4, 250 mM mannitol, 0.5 mM EGTA, 0.1% BSA), supplemented with protease inhibitors, and homogenized. The homogenate was centrifuged at 800× g for 10 minutes at 4°C, and the supernatant was then centrifuged at 12,000× g for 30 minutes at 4°C. The resulting pellet (mitochondrial fraction) was resuspended in buffer M and the final supernatant represented the cytosolic fraction. Protein concentration was determined as previously indicated. Aliquots of both fractions (cytosolic and mitochondrial) were used in Western blotting analysis for the cytochrome *c* localization.

2.10. Measurement of SOD Activity. SH-SY5Y cells were plated at a density of 2×10^6 cells/plate (75 cm^2) and, after one-day plating, 150 μM diclofenac was added to the cultures. Cells were then collected at different times as previously indicated and then centrifuged at 300× g at 4°C for 5 minutes, washed once with PBS, and finally resuspended in 50 mM potassium phoshate, pH 7.8, containing 1 mM EDTA. The cellular suspension was sonicated, centrifuged at 20,000× g at 4°C for 30 minutes, and after protein quantitation, the supernatant was used for an activity gel assay. In particular, aliquots of cell extracts (50 μg) were fractionated on a 10% polyacrylamide gel and SOD activity was evaluated as previously described [25]. This method is based on the inhibitory effect of SOD on the reduction of nitro-blue tetrazolium by the superoxide anions generated by the photochemical reduction of riboflavin; SOD is visualized as a colourless band over a blue background.

2.11. Statistical Analysis. All results are presented as histograms and data are the average ± SD of at least three independent measurements. In particular, data were analyzed by one-way ANOVA, and differences were considered significant when the corresponding *P*-values were $< .05$ in the Bonferroni's post-hoc test.

3. Results

3.1. Diclofenac-Induced Apoptosis in the Human Neuroblastoma Cell Line SH-SY5Y. To evaluate whether diclofenac had cytotoxic effects on neuronal cells, the human neuroblastoma cell line SH-SY5Y was incubated for 24 hours with increasing concentrations of diclofenac. Propidium iodide incorporation followed by flow cytometric analysis showed a dose-dependent increase of apoptotic nuclei with sub-diploid DNA content (Figure 1(a)). The cytosolic enzyme lactate dehydrogenase (LDH) has been already used as a marker of cytotoxic injury [11]. Indeed, a significant increase of LDH activity is typically found in culture media of cells undergoing a disruption of plasma membrane. To this aim, we have assayed the LDH activity in the culture media of SH-SY5Y cells incubated up to 48 hours with increasing concentrations of diclofenac. No significant release of the intracellular LDH was observed up to 150 μM diclofenac (not shown), which was taken as the maximal concentration of this compound not causing a significant cytotoxic effect (MNTC). To analyse the time-dependent increase of apoptosis, SH-SY5Y cells were exposed to 150 μM diclofenac and analysed at different times. As shown in Figure 1(b), the



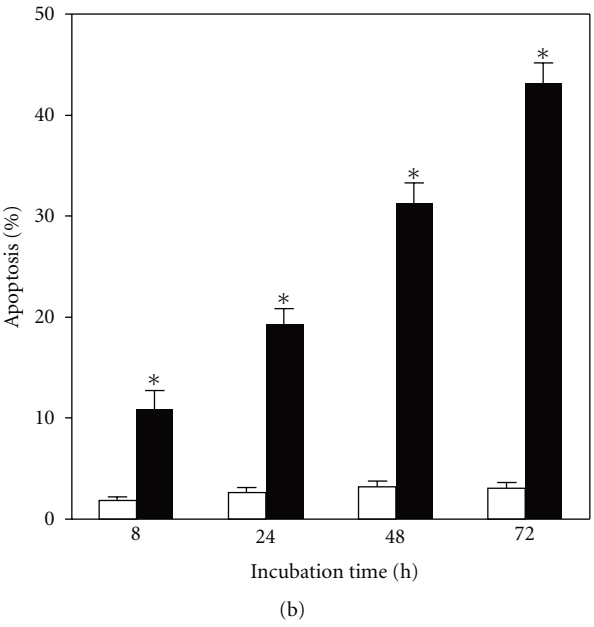


Figure 1: Diclofenac-induced apoptosis in SHSY-5Y cells. (a) SH-SY5Y cells were incubated with the indicated concentrations of diclofenac and apoptosis was evaluated after a 24-hour drug treatment. (b) SH-SY5Y cells were treated with vehicle alone (white bars) or 150 μM diclofenac (black bars) and apoptosis was evaluated after the indicated incubation times. The percentage of apoptosis was measured as described in Section 2.4; $^*P < .01$ compared to untreated cells.

apoptotic process was already evident after 8-hour treatment and progressively increased up to 72 hours.

3.2. Effect of Diclofenac on the Intracellular ROS Level. It is known that NSAIDs alter the redox state of different cell types through an enhancement of intracellular ROS levels

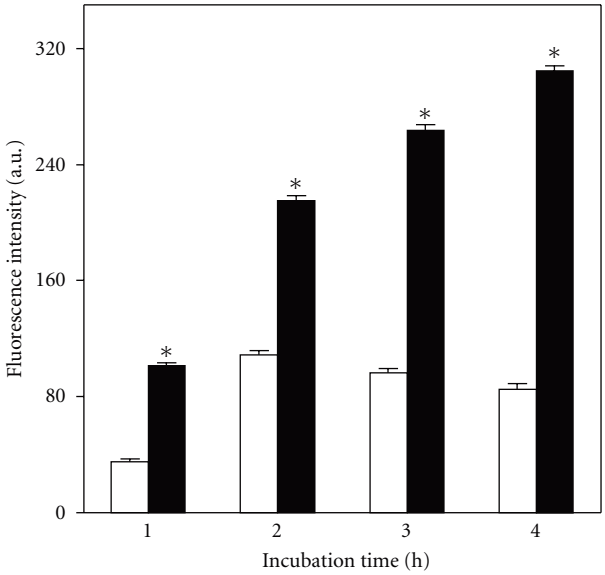


Figure 2: Effect of diclofenac on the intracellular ROS level. SH-SY5Y cells were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for the indicated times. The intracellular ROS level was evaluated through the usage of the fluorescent probe DCFH-DA, as described in Section 2.5. Fluorescence intensity was reported as Arbitrary Units (AU); $^*P < .01$ compared to untreated cells.

[9–11]. Therefore, we have decided to investigate whether diclofenac affects the intracellular ROS levels also in SH-SY5Y cells, using the fluorescent probe DCFH-DA. Indeed, when these cells were incubated with diclofenac and DCFH-DA at different times, an early increase of the ROS level was detected with respect to control cells; furthermore, ROS production progressively continued at least up to 4 hours (Figure 2).

3.3. Effect of Diclofenac on SOD2 Levels. As SOD2 is the primary antioxidant enzyme in mitochondria protecting cells from oxidative injuries, we have evaluated the role exerted by this enzyme in SH-SY5Y cells in the course of diclofenac treatment. To this aim, the dose- and time-dependent effects of this drug on SOD2 protein levels of SH-SY5Y cells were analysed in the experiments reported in Figure 3. When these neuronal cells were incubated for 24 hours with increasing diclofenac concentrations, a dose-dependent decrease of the SOD2 protein level was observed, as evaluated by Western blotting using antibodies against human SOD2 (Figure 3(a)). A similar picture emerged when SH-SY5Y cells were incubated with 150 μM diclofenac and analysed at different times. The decrease of SOD2 levels (Figure 3(b)) was evident after 24-hour treatment and increased at 48 hours.

To investigate the possibility that the reduction of the SOD2 protein levels corresponded also to a decrease in SOD activity, the protein extracts obtained from cells incubated with 150 μM diclofenac were analysed at different times using a SOD activity gel, which allows the detection of the activity

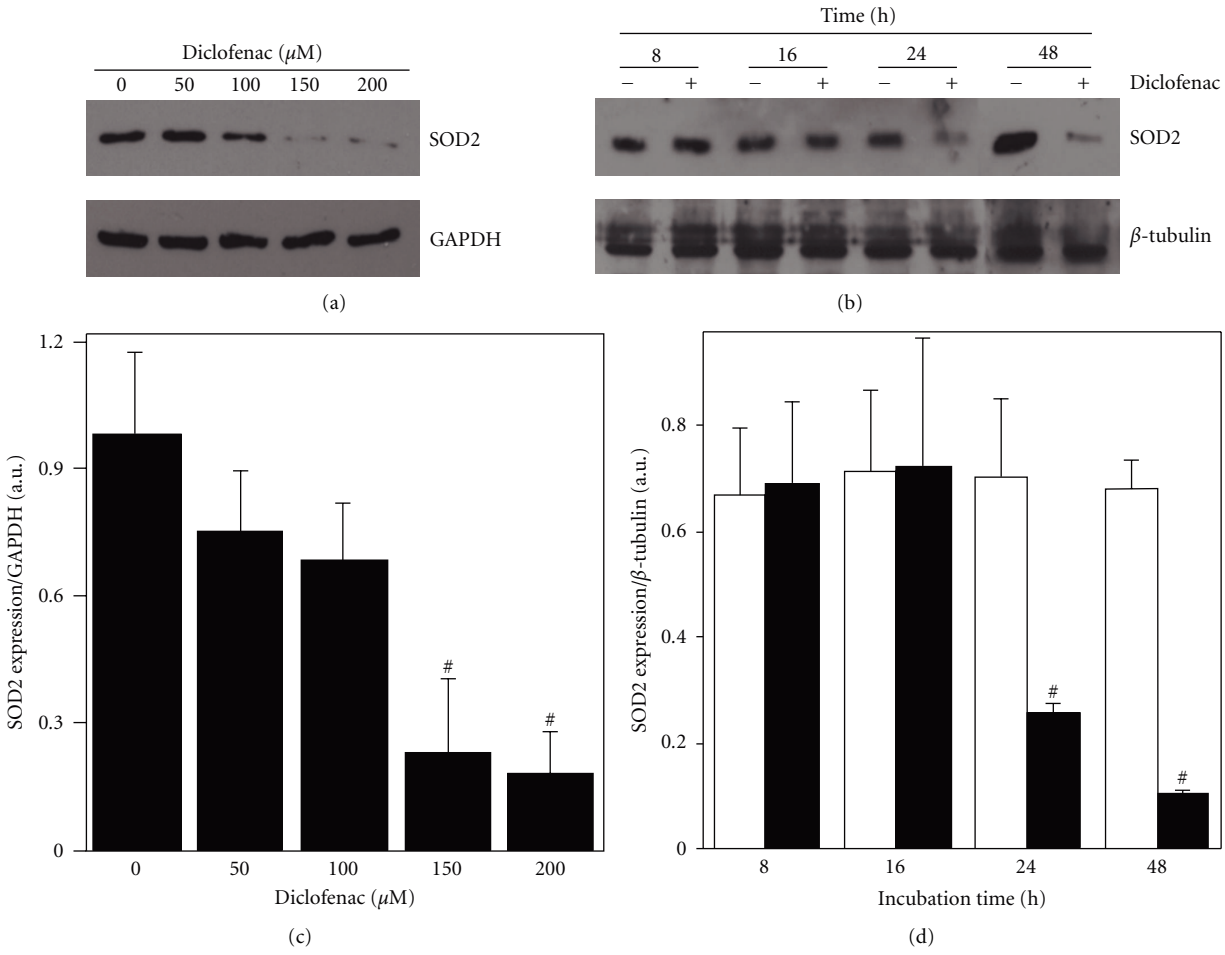


Figure 3: Effect of diclofenac on SOD2 protein levels. (a) Dose dependence. SH-SY5Y cells were incubated with the indicated concentrations of diclofenac for 24 hours. The SOD2 protein level was evaluated by immunoblotting; as a loading control, the same filter was probed with anti-GAPDH antibody. (b) Time dependence. SH-SY5Y cells were incubated with vehicle alone or 150 μM diclofenac at the indicated times and treated as indicated in (a). β-tubulin was used as house-keeping control. (c, d) Densitometry evaluation of three independent experiments carried out as in (a) and (b), respectively; $^{\#}P < .05$ compared to untreated cells.

of both mitochondrial SOD2 and cytosolic SOD1. Interestingly, the SOD2 activity decreased in a time-dependent manner (Figure 4), thus indicating that the reduction of the enzyme level caused a lower efficiency in the defence against the superoxide anions. Vice versa, the activity of SOD1 was not affected by the diclofenac treatment (not shown).

We also evaluated whether the diclofenac-induced decrease of SOD2 levels/activity depends on a down-regulation of the corresponding mRNA. To this aim, SH-SY5Y cells were incubated in the presence or in the absence of 150 μM diclofenac for 4, 8, and 24 hours. In order to evaluate a possible regulation of the enzyme at transcriptional level, after RNA extraction, a cDNA was obtained by reverse transcriptase and used as a template in a PCR realised in the presence of specific oligonucleotides for SOD2. The data presented in Figure 5 indicate that the diclofenac treatment did not significantly alter the mRNA levels of SOD2 at all times investigated. A similar picture with proportionally lower signals emerged when dilutions of the cDNA template were used in the RT-PCR assay (not shown). These results, together with the low number of amplification cycles used, confirm that the employed PCR conditions fell in the linear dose-response range.

3.4. Effect of a Heterologous Thioredoxin on the Antioxidant Power of Diclofenac-Treated Cells. Thioredoxin (Trx), a small (12 kDa) and ubiquitous protein involved in many cellular functions, is a potent disulphide oxidoreductase controlling the reduced state of intracellular proteins. The crucial antioxidant power of Trx also takes advantage of its ability to cross the cellular membrane; in fact, evidences have been reported that Trx can be released from cells [26–28] and even enter a living cell [29, 30]. Moreover, endogenously produced or exogenously added Trx increases the SOD2 expression in lung carcinoma [31] or neuronal cells [32]. To this aim, the effect of TrxA2 from the hyperthermophilic archaeon *Sulfolobus solfataricus* (*Ss*Trx)

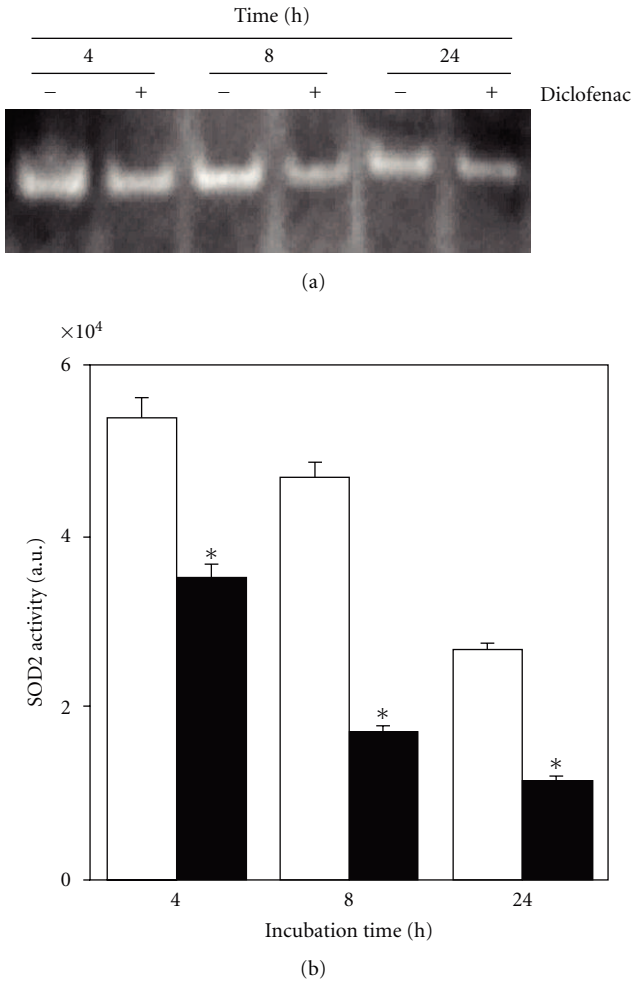


Figure 4: Effect of diclofenac on SOD2 activity. (a) SH-SY5Y cells were treated with vehicle alone or 150 μM diclofenac for the indicated times. Total cell extracts were fractionated on a nondenaturing polyacrylamide gel and SOD2 activity was evaluated as described in Section 2.10. (b) Densitometry evaluation of three independent experiments; $^*P < .01$ compared to untreated cells.

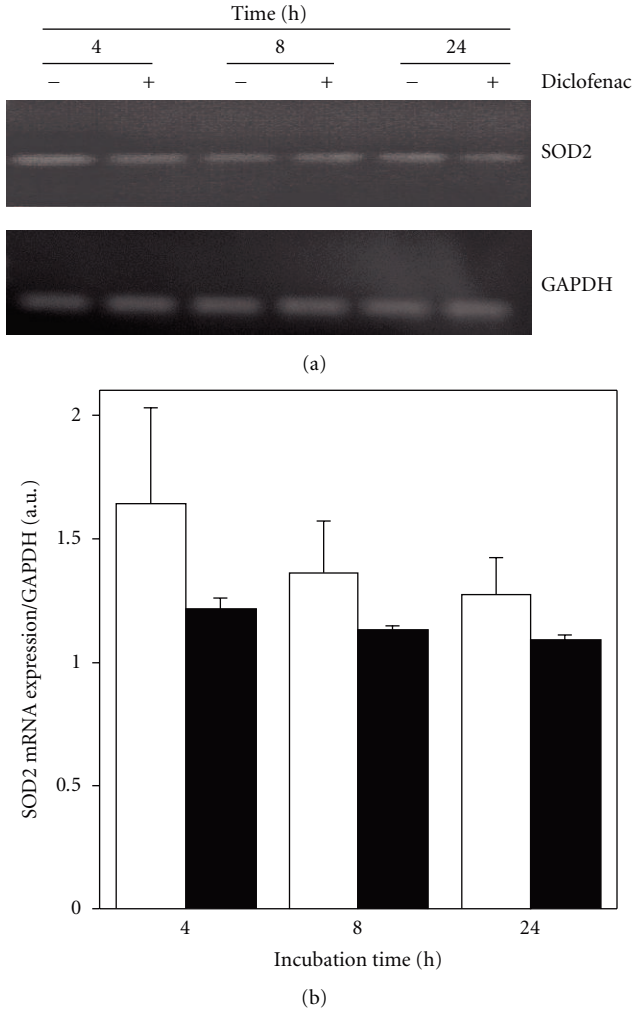


Figure 5: Analysis of SOD2 mRNA level in SH-SY5Y cells treated with diclofenac. (a) Total RNA extracts from SH-SY5Y cells, incubated with vehicle alone or 150 μM diclofenac for the indicated times, were amplified by RT-PCR as indicated in Section 2.6 and analysed on a 1% agarose gel. As a control, the amplification of GADPH from the same extracts was carried out. (b) Densitometry evaluation of three independent experiments.

on the reduction of SOD2 levels caused by diclofenac was evaluated (Figure 6). When SH-SY5Y cells were treated with 150 μM diclofenac, the SOD2 protein level was significantly reduced. The addition of increasing concentrations of *Ss*Trx allowed a restoration of the SOD2 level, thus counteracting the effect of diclofenac. In particular, in the presence of 10 μM *Ss*Trx, the amount of SOD2 was even higher compared to untreated cells. In a control experiment, the addition of 10 μM *Ss*Trx did not cause any significant variation of the SOD2 level in the absence of diclofenac (not shown).

The possible protective effect of *Ss*Trx on the diclofenac-induced apoptosis was also evaluated. As shown in Figure 7, the addition of *Ss*Trx partially reverted the programmed cell death provoked by the drug. In particular, in the presence of 10 μM *Ss*Trx, the apoptosis of the diclofenac-treated cells was reduced at all times investigated.

3.5. Effect of Diclofenac on Mitochondrial Membrane Potential and Cytochrome c Localization. Loss of the membrane mitochondrial potential occurs as an early event during the apoptosis induced by specific stimuli in some cellular systems [33]. To better evaluate the molecular mechanisms underlying the diclofenac-induced apoptosis in SH-SY5Y cells, the efficiency of the mitochondrial function was evaluated through measurements of its membrane potential, using the fluorescent probe R123. It is known that this compound crosses the mitochondrial membrane and accumulates into the matrix, only when the transmembrane potential is preserved; therefore, in case of a loss of the membrane potential, the R123 fluorescence undergoes a significant reduction [34]. As shown in Figure 8, the mitochondrial incorporation of R123 in SH-SY5Y cells treated with 150 μM diclofenac underwent a significant reduction compared to

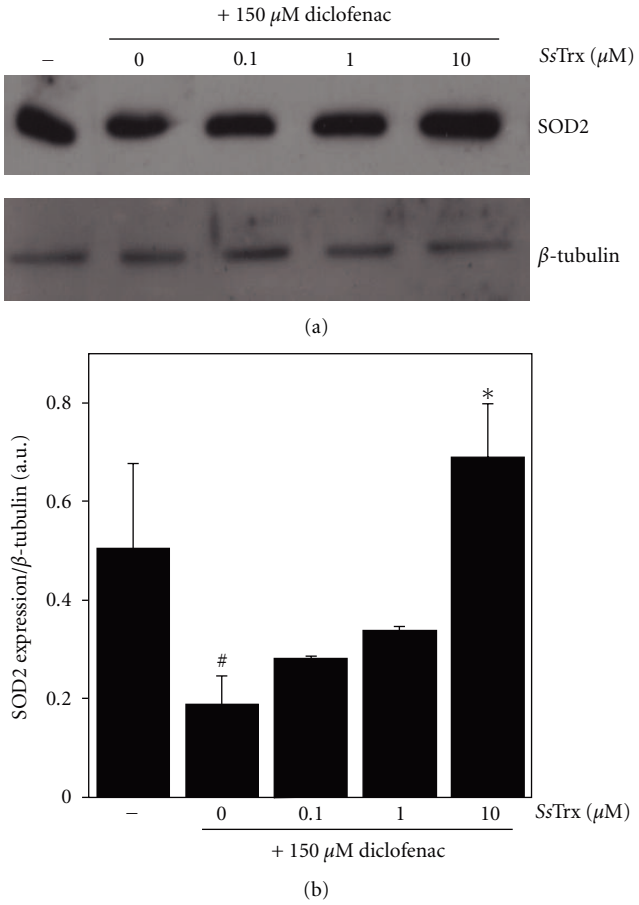


FIGURE 6: Effect of a heterologous thioredoxin on the reduced SOD2 protein level caused by diclofenac. (a) SH-SY5Y cells were incubated for 24 hours in the absence or in the presence of 150 μM diclofenac plus the indicated concentrations of *Ss*Trx. The SOD2 protein level was evaluated by immunoblotting. As a loading control, the same filter was probed with an anti-β-tubulin antibody. (b) Densitometry evaluation of three independent experiments; $^{\#}P < .05$ compared to untreated cells; $^{*}P < .01$ compared to diclofenac-treated cells.

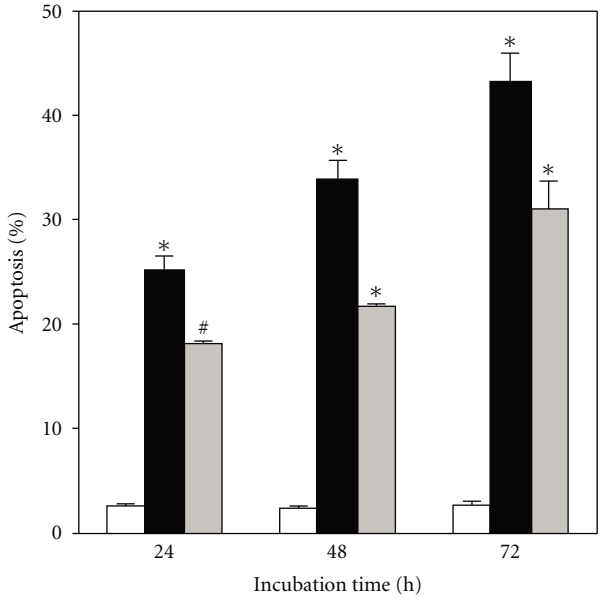


FIGURE 7: Partial reversion of the diclofenac-induced apoptosis by *Ss*Trx. SH-SY5Y cells were incubated for the indicated times in the absence (white bars) or in the presence of 150 μM diclofenac without (black bars) or with 10 μM *Ss*Trx (gray bars). The percentage of apoptosis was measured as described in Section 2.4; $^{*}P < .01$ compared to untreated cells or diclofenac treated cells; $^{\#}P < .05$ compared to diclofenac-treated cells.

that measured on untreated cells. This hypopolarization of the mitochondrial membrane caused by diclofenac became evident after 2-hour treatment and significantly increased with the incubation time.

A deeper insight on the involvement of mitochondria in the diclofenac-induced apoptosis was realised with a Western blotting analysis aimed at the evaluation of the cytochrome *c* release from mitochondria. In particular, the detection of cytochrome *c* was carried out on both cytosolic and mitochondrial protein fractions from SH-SY5Y cells incubated in the absence or in the presence of 150 μM of diclofenac at different times (Figure 9). Interestingly, the cytochrome *c* was already present after a 8-hour incubation mainly in the cytosolic fraction of diclofenac-treated cells; moreover, the amount of this mitochondrial marker in the cytosol significantly increased after a 24-hour treatment (Figure 9(a)). This behaviour was confirmed by the concomitant analysis on the mitochondrial fraction, where a

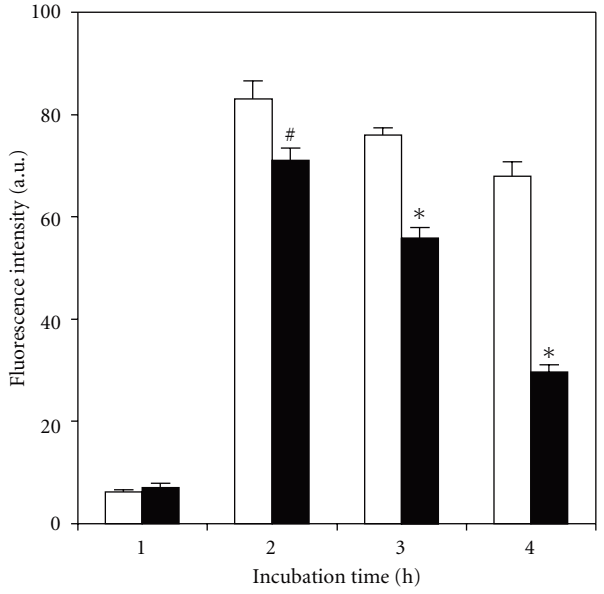


FIGURE 8: Reduction of the mitochondrial membrane potential in the presence of diclofenac. SH-SY5Y cells were incubated with vehicle alone (white bars) or 150 μM diclofenac (black bars) for the indicated times. Mitochondrial membrane potential was evaluated through the incorporation of the fluorescent probe R123 as described in Section 2.7. $^{*}P < .01$ and $^{\#}P < .05$, compared to untreated cells.

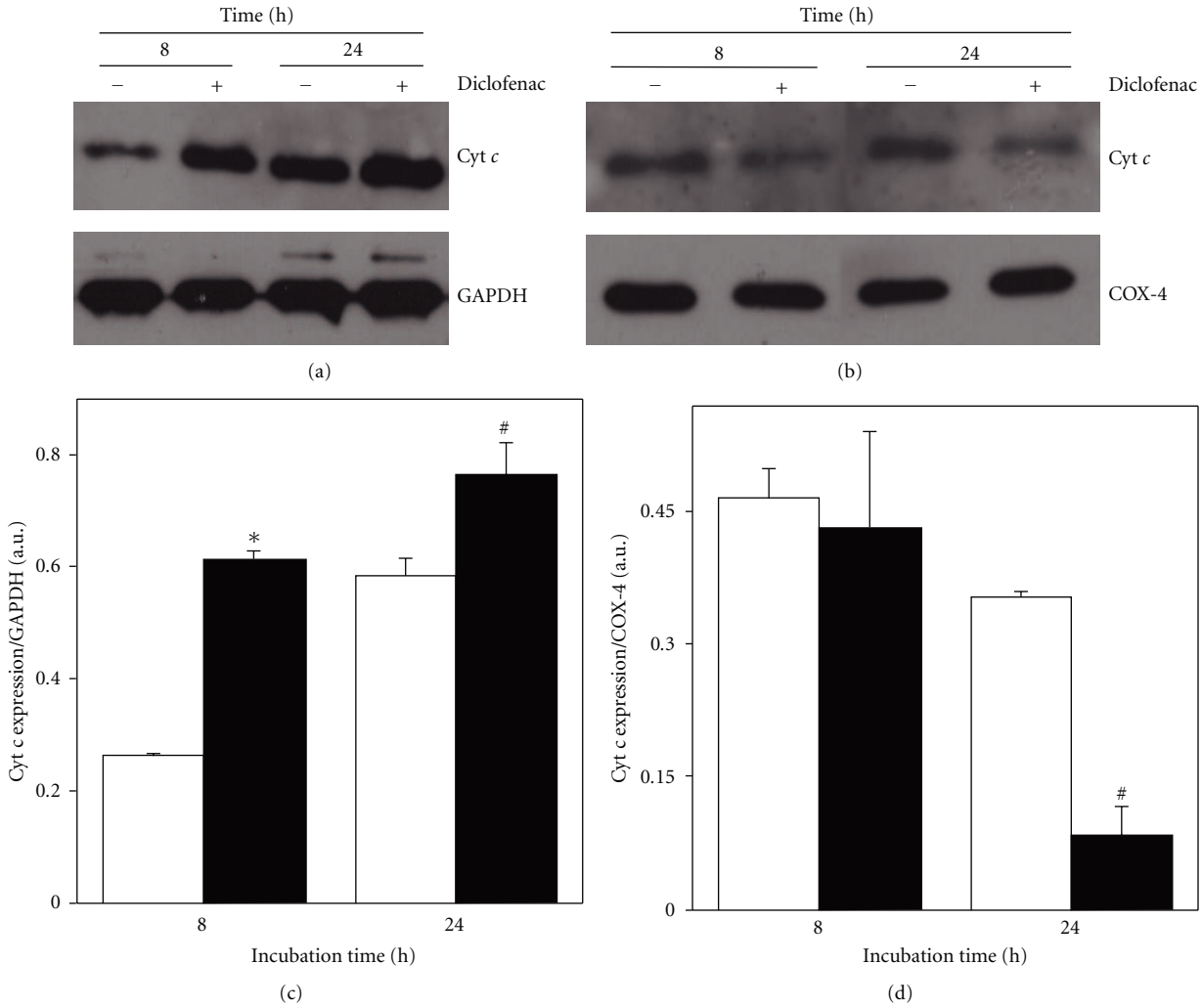


Figure 9: Effect of diclofenac on the subcellular distribution of cytochrome *c*. Cytosolic and mitochondrial fractions were prepared from SH-SY5Y cells incubated in the absence or in the presence of 150 μM diclofenac for the indicated times. Distribution of cytochrome *c* (Cyt *c*) in cytosolic (a) and mitochondrial (b) fraction was evaluated by immunoblotting. The same filters were also probed with anti-GAPDH (a) or anti-COX-4 (b) antibodies as loading control in the cytosolic or mitochondrial extracts, respectively. (c, d) Densitometry evaluation of three independent experiments carried out as in (a) and (b), respectively; $^{*}P < .01$ and $^{\#}P < .05$ compared to untreated cells.

reduction of the cytochrome *c* level was observed in the diclofenac-treated cells (Figure 9(b)). These data indicate that cytochrome *c* is released from the mitochondria, as a consequence of the diclofenac treatment of SH-SY5Y cells.

4. Discussion

In this paper we describe the effects of diclofenac on cultures of the neuroblastoma cell line SH-SY5Y, thus extending to this neuronal cell our knowledge on the possible toxicity of this drug, already reported for gastric, hepatic, or renal cells. Indeed, we present evidence that diclofenac induces apoptosis through a modulation of the mitochondrial function, associated to an alteration of the redox homeostasis.

It is known that apoptosis is the primary cell death induced by drugs [35] and that this process is mainly mediated by the mitochondrial pathway [36, 37]. Here we show that diclofenac induces apoptosis of SH-SY5Y cells in a time- and concentration-dependent manner, thus suggesting that this compound is somehow toxic even for a neuronal-type cell. Previous pharmacokinetic studies demonstrated that the blood level of diclofenac, on the basis of the therapeutic doses used in patients (50–150 mg/day), ranges between 10 and 30 μM [38]. However, in some circumstances the local concentration of the drug might increase. In particular, diclofenac concentration increases as a consequence of long-term treatments, overdosing, limited clearance, and so forth, as previously reported [39–42]. For these reasons, in our in vitro studies we have used a diclofenac concentration of 150 μM, because this value corresponded to the maximal

concentration of this compound not causing cytotoxicity, as determined by cytotoxicity assays on SH-SY5Y cells. In the light of the large therapeutic usage of diclofenac, these data are more relevant because of the known ability of this drug to cross the blood-brain barrier [43]. Apoptosis is regulated by many signals and metabolic events, and the cellular redox state plays a critical role in this process. Indeed, several data showed that ROS, mainly produced by mitochondria, are involved in the programmed cell death through an induction of the oxidative stress [44]. In particular, an increased ROS level frequently represents a triggering event upstream of the mitochondrial membrane depolarization, cytochrome *c* release, caspase activation, and nuclear fragmentation [45]. An alteration of the ROS levels was already demonstrated in diclofenac-induced apoptosis in gastric and renal cells [9, 10]. Also in our neuronal cell system diclofenac provokes an early and significant increase of the intracellular ROS levels.

These results prompted us to evaluate the downstream effects of altered ROS levels in the course of diclofenac treatment of SH-SY5Y cells. Under this concern, SOD2 represents the major antioxidant enzyme in mitochondria, where the intense cellular respiration may produce a large amount of ROS. Therefore, an efficient SOD2 activity is required to counteract the mitochondrial dysfunction induced by an oxidative stress, which could lead to the programmed cell death usually observed in various disease contexts [46]. The antiapoptotic role of SOD2 is even demonstrated by the resistance to the radiation-induced damage reported for cell lines overexpressing SOD2 [47]. Our data on SH-SY5Y cells show that diclofenac impairs SOD2 functions, thus suggesting that this enzyme is involved in the apoptotic mechanism induced by the drug. In particular, this process is associated to a significant and concomitant reduction of both protein level and enzymatic activity of SOD2, whereas RT-PCR experiments showed that the corresponding mRNA levels are not affected by the diclofenac treatment. Therefore, a transcriptional regulation of the SOD2 gene by diclofenac could be excluded; probably, an increased degradation of the protein levels could explain the reduced activity of the enzyme. The behaviour of SOD2 in the diclofenac response was compared with the effects reported for other compounds in various experimental systems. For instance, stautosporine, a protein kinase inhibitor, did not affect the mRNA levels of SOD1 and SOD2, but decreased protein and activity levels of both enzymes [48]. Other authors reported that SOD2 is degraded by caspases in Jurkat T cells, following oligomerization of the Fas receptor [20]. On the other hand, in rat astrocytes the lipopolysaccharide induced an increase of SOD2 mRNA, but not of the SOD2 protein [49]. These observations confirm the regulation of SOD2 functions following a drug treatment; however, the differences observed among the various experimental systems indicate that the SOD2 response is a multifactorial process only poorly clarified.

It is known that the induction of SOD2 can be mediated by various macromolecules, such as interleukyn-1 [50], lipopolysaccarides [51], and tumor necrosis factor-α [52]; furthermore, thiol-reducing agents can affect SOD2 biosynthesis, as demonstrated by the enhanced SOD2 expression caused by thioredoxin, a potent disulfide oxidoreductase [31]. In this paper, the addition of a heterologous thioredoxin to diclofenac-treated cultures of SH-SY5Y led to an enhancement of the SOD2 levels, as well as to a reduction of the apoptosis. This result confirms the involvement of SOD2 in the apoptotic processes induced by the drug and suggests that an archaeal thioredoxin is active also in human cells. Furthermore, we can speculate on a possible functional interaction between heterologous components of the thioredoxin system, because purified archaeal *Ss*Trx was added in its oxidised inactive form; its conversion to the reduced active form involves a reaction putatively catalysed by the human thioredoxin reductase.

The increased ROS level, the low functionality of the mitochondrial antioxidant enzyme SOD2, together with a partial recovery of SOD2 properties by a heterelogous thioredoxin, strongly suggest the involvement of mitochondria in the diclofenac-induced apoptosis of SH-SY5Y cells. This hypothesis was confirmed by measurements of the mitochondrial membrane potential. Indeed, an excessive production of ROS and a decrease in SOD2 levels contribute to the mitochondrial dysfunction. In particular, after a significant loss of the mithocondrial membrane potential, apoptosis-inducing factors are released from the mitochondria, thus leading to the activation of the caspase cascade, and ultimately to nuclear condensation [44, 45, 53, 54]. Also in our system, the diclofenac treatment induces an early mitochondrial hypopolarization, correlated to an increase of the intracellular ROS levels, both events representing typical features of the onset of mitochondrial apoptosis. Another crucial marker of the intrinsic apoptosis is the cytocrome *c* release from the mitochondria, as a consequence of membrane depolarization. Our data showed that cytochrome *c* translocates from the mitochondria to the cytosol during the diclofenac treatment of SH-SY5Y cells. This finding, together with the mitochondrial membrane depolarization, provides a more direct link between mitochondria and diclofenac-induced apoptosis, thus confirming that the programmed cell death in SH-SY5Y follows the mitochondrial pathway.

The results obtained in this work could be relevant for a deeper insight on the therapeutic usage of diclofenac, pointing to the oxidative damage related to its cytotoxic effect. In particular, the involvement of the main antioxidant mitochondrial enzyme in the apoptotic process may suggest the use of SOD2 small interfering RNA in combination with diclofenac, in order to improve the treatment of cancer, such as neuroblastoma. On the other hand, diclofenac, because of its ability to alter the cellular redox state of neuronal cells, could be considered a neurotoxic compound. Under this concern, previous studies showed that diclofenac and indomethacin, another NSAID, enhance the effects of some neurotoxins on PC12 cells [55] and that diclofenac inhibits the proliferation and differentiation of neuronal stem cells [8]. Our results on the protective effects of *Ss*Trx open some perspectives on the possible counteraction of the side effects caused by diclofenac.

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