UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA
BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE
XXIV CICLO

Apoptosis induction in different melanoma cell lines by NEMO Binding Domain (NBD) peptide treatment

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Anno Accademico 2010/2011

Alla mia famiglia
ed a Giampaolo

Ringraziamenti

Nell'accingermi a concludere la stesura di questo lavoro di tesi, sento il desiderio di esprimere delle sincere parole di ringraziamento per delle persone speciali a cui sono legata da un profondo affetto e stima, che hanno condiviso con me questa intensa ed entusiasmante esperienza. Il mio pensiero va innanzitutto al mio relatore, professoressa Maria Rosaria Faraone Mennella, per avermi insegnato ad affrontare la ricerca con serietà e dedizione, ma anche con la giusta dose di spirito. Grazie prof per i momenti di crescita culturale e per il bagaglio di esperienza e capacità che, con una umanità quasi materna, lei mi ha quotidianamente trasmesso.

Ringrazio con uguale decisione la dottoressa Anna De Maio, con il quale ho assaporato il dolce piacere dello scoprire e del farlo con entusiasmo e passione, per quanto sia stato un punto di riferimento con le sue indicazioni e la sua serietà. Grazie Anna per la disponibilità e i consigli preziosi che hanno quotidianamente guidato lo svolgimento di questo lavoro.

Grazie alla professoressa Benedetta Farina per l'affetto, la disponibilità offerta ed i suoi preziosi consigli.

Un ringraziamento particolare va alla mia cara amica Elena con la quale ho condiviso le tappe di questo percorso. Grazie per l'amicizia, la sincerità del tuo affetto e per i tanti momenti di allegria e spensieratezza.

Desidero ringraziare i ragazzi che hanno svolto la tesi sperimentale con me e che hanno contribuito a rendere questi anni lieti Consuelo, Elena, Lucia, Anna Rita, Rossella D., Lidia, Mariagrazia, Maddalena e Rossella insieme a Teresa, Maria.

Grazie a Silvia con la quale, nonostante io abbia lavorato per poco tempo, si è instaurata una bella amicizia.

Desidero ringraziare con immenso affetto le persone più importanti della mia vita a cui dedico questo lavoro. In particolare i miei genitori e le mie sorelle Daniela e Stefania per la capacità che avete di rassicurarmi, per avermi sostenuto con l'amore e la stima in ogni momento della mia vita, grazie per avermi insegnato quanto sia importante credere nei propri mezzi e non mollare; grazie a Giampaolo per aver condiviso con me ansie e successi e per l'amore ed il sostegno che mi dimostri.

Riassunto

Il melanoma rappresenta la forma più aggressiva di cancro della pelle e si origina per trasformazione neoplastica dei melanociti. L'esposizione ai raggi UV e la suscettibilità genetica rappresentano i principali fattori di rischio per lo sviluppo di questa patologia, che risulta particolarmente resistente a diversi tipi di trattamenti terapeutici e chemioterapici. In quest'ottica, negli ultimi anni, sono stati identificati nuovi pathways di segnalazione cellulare, coinvolti nella tumorigenesi, al fine di individuare nuovi bersagli per le terapie.

Nella progressione del melanoma è noto che il fattore di trascrizione nucleare NF-kB risulta costitutivamente attivato in diverse linee di melanoma ed in grado di regolare l'espressione e la funzione di un ampio spettro di geni coinvolti nella risposta immediata ad agenti patogeni, nell'infiammazione, nella proliferazione e sopravvivenza cellulare e nell'apoptosi.

Alla luce di queste conoscenze, pertanto, si è ipotizzato che la sua inattivazione potesse indurre le cellule trasformate verso morte programmata. A tale scopo, cellule di melanoma umano A375 sono state incubate per tempi diversi con differenti concentrazioni di NBD-peptide, un peptide di cui erano già note le proprietà antinfiammatorie.

Nell'ambito di questo studio la poli(ADPR)polimerasi1 (PARP-1) è stata scelta come marcatore di apoptosi. E' noto, infatti, che la PARP-1 (113kDa) è substrato della caspasi 3, che opera la sua proteolisi in due frammenti stabili ed inattivi (89kDa e 24kDa). I risultati ottenuti hanno evidenziato che il trattamento con NBD-peptide induce una inibizione della proliferazione cellulare dipendente dalle concentrazioni di peptide utilizzate.

La conferma che le cellule trattate muoiono per apoptosi è stata ottenuta sia mediante citometria a flusso (FACS analysis) che attraverso le analisi di espressione e di attività della PARP-1. L'analisi al FACS ha, infatti, mostrato che nelle cellule trattate si verifica attivazione della caspasi-3, responsabile della parziale frammentazione ed inattivazione della PARP-1 osservate rispettivamente mediante Western blotting e misura di attività enzimatica.

Il saggio EMSA (electrophoretic mobility shift assay) effettuato per determinare l'attività sia di NF-kB, che del complesso IkB chinasi (IKK), hanno confermato l'ipotesi che il peptide induce apoptosi attraverso la modulazione del fattore di trascrizione nucleare. Tale modulazione è conseguenza del blocco dell'attività del complesso IKK, responsabile dell'attivazione dil NF-kB. E' noto, infatti, che quest'ultimo, in forma non attivata, non può traslocare nel nucleo, dove è responsabile della trascrizione di diversi tipi di geni, tra i quali quelli antiapoptotici.

I dati preliminari suggeriscono anche che la parziale inattivazione della PARP-1 potrebbe contribuire al mantenimento del processo apoptotico.

Poiché è stato dimostrato che anche i livelli di poli-ADP-ribosio (PAR) giocano un ruolo cruciale sia nella vita che nella morte cellulare, nella seconda fase di ricerca sono stati condotti studi atti a verificare se nelle cellule di melanoma il trattamento con NBD-peptide modifica il turnover del poli-ADP-ribosio. A tale scopo è stata studiata, oltre alla sintesi del poli-ADP-ribosio, anche la sua degradazione, della quale è responsabile la poli(ADPR)glicoidrolasi (PARG).

Nelle cellule di melanoma A375 trattate e non con NBD peptide, è espressa la stessa isoforma dell' enzima PARG con un peso molecolare di circa

75kDa, a localizzazione nucleare. L'attività dell'enzima è significativamente più elevata nelle cellule di melanoma non trattate, nelle quali è garantito un attivo "turnover" di PAR ed è favorita la sopravvivenza della cellula tumorale.

Nelle cellule trattate, i più bassi livelli di attività della PARG potrebbero essere spiegati considerando che la sua attività è influenzata sia dalla lunghezza del PAR che dalla sua concentrazione intranucleare. La parziale inattivazione della PARP-1 indica, infatti, che i livelli di PAR nelle cellule trattate sono più bassi di quelli prodotti nelle cellule di melanoma. Inoltre, in seguito ad isolamento del PAR, è stato osservato che il polimero proveniente dalle cellule A375 di melanoma trattate è più corto e non ramificato di quello delle cellule non trattate.

In conclusione possiamo ipotizzare che nelle cellule trattate, dove il processo apoptotico è attivo, i bassi livelli di attività PARP-1 producono livelli di PAR bassi e non tossici, i quali combinati con una bassa attività dell'enzima PARG potrebbero consentire alla cellula di mantenere lo stato apoptotico piuttosto che andare in necrosi.

Nell'ultima fase della ricerca è stata confermata l'efficacia del peptide NBD anche su altre sei linee di melanoma con diverse mutazioni geniche.

L' impiego del peptide NBD, pertanto potrebbe rappresentare un innovativo ed efficiente strumento per la cura del melanoma. A differenza dei comuni farmaci utilizzati per l'inibizione dell'attività di NF-kB, inoltre, NBD-peptide non determina la totale inattivazione del fattore trascrizionale, consentendone un ruolo fisiologico nell'immunità, nell'infiammazione e nell'omeostasi cellulare.

Summary

Melanoma is the most aggressive form of skin cancer that originates from neoplastic trasformation of melanocyte cells. Important predisposing factors are considered UV exposure and genetic susceptibility. Due to the complex nature of the disease, metastatic melanomas have proven to be typically resistant to different therapy and chemotherapy treatments. On this basis, recently, key signalling pathways that are important in promoting melanoma tumourigenesis, have been identified thus providing dynamic targets for therapy.

The NF-kB is a well known transcriptional nuclear factor involved in melanoma progression. It is constitutively activated in several melanoma cell lines and regulates the expression and function of several genes involved in immediate early pathogen response, in inflammation, cell proliferation and survival and in apoptosis.

Based on this knowledge, it was hypothesized that its inhibition could represent a new way to induce programmed death in tumoral cells.

Therefore, A375 melanoma cells were incubated with different concentrations of NBD-peptide at different times. The anti-inflammatory activity of this peptide was already known.

In this study, PARP-1 was used as marker of apoptotic process. In fact, it is a substrate of caspase-3, responsible for its cleavage in two stable and inactive fragments (89kDa and 24kDa). The results showed that the NBD peptide treatment induces a concentration-dependent inhibition of melanoma cell proliferation.

FACS analysis (flow cytometry) using the monoclonal antibody (mAb) PE-conjugated anti-human-active caspase-3, together with PARP-1 expression

and activity analyses, confirmed that the treated cells die by apoptosis. FACS analysis showed that in treated cells, a concentration-dependent activation of caspase-3 occurs. Partial fragmentation and inactivation of PARP-1, is also observed by western blotting and enzimatic assay, respectively.

EMSA assay (electrophoretic mobility shift assay) carried out to study both NF-kB and IKK kinase complex activities, confirmed the hypothesis that the peptide induces apoptosis by modulating activity of this transcriptional nuclear factor. Such modulation is due to the block of IKK complex activity, responsible of NF-kB activation. In fact, it' s known that the latter, inactive, cannot translocate into nucleus, where it modulates transcription of different genes among which the antiapoptotic genes.

Preliminary data also suggest that partial PARP-1 inactivation could allow the maintenance of apoptotic process. PARP-1, in fact, is one of the main consumers of intracellular energy.

Since it was demonstrated that poly-ADP-ribose (PAR) levels play an important role both in cellular survival and death, in the second part of the present research it was studied whether NBD peptide treatment influences poly-ADP-ribose turnover. To this aim, beside poly-ADP-ribose synthesis, its degradation, by poly(ADPR)glycohydrolase (PARG) was studied.

In both NBD treated and untreated A375 melanoma cells, the same PARG isoform is expressed (about 75kDa), localized into the nucleus.

Its activity is more high in untreated melanoma cells, in which an active PAR turnover is guaranteed and tumoral cell survival occurs.

In treated cells, the lowest activity of PARG could depend on both length and intranuclear concentration of PAR. The partial PARP-1 inactivation

suggests that PAR levels in treated cells are lower than those produced in melanoma cells. Moreover, a comparison of PAR isolated from treated cells and that from melanoma cells, indicates that the former is unbranched and shorter.

In conclusion, it is possible to hypothesize that in treated A375 cells, where the apoptotic process occurs, the low PARP activity produces low and no toxic PAR levels that combined with a low PARG activity, might allow the cells to maintain the apoptotic state rather than undergo necrosis.

In the last part of research the efficience of NBD peptide was also tested on other seven melanoma cell lines with different gene mutations. So, this peptide might represent a new and efficient drug in melanoma care. In addition, respect to common drugs used to inhibit NF-kB activity, NBD-peptide doesn't block nuclear factor activity completely, allowing its physiological roles in immunity, inflammation and cellular homeostasis.

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INTRODUCTION

1.1 Poly(ADP-ribosyl)ation reaction and PARP enzymes

Poly(ADP-ribosyl)ation is a post-translational modification of proteins involved in a wide range of molecular and cellular processes, including DNA damage detection and repair, chromatin modification, apoptosis, proliferation, differentiation and mitotic apparatus function (Hottiger *et al*., 2010).

These processes are critical for many physiological and pathophysiological outcomes (genome maintenance, carcinogenesis, aging, inflammation, and neuronal function) (Mègnin. *et al*., 2010., Rupinder *et al*., 2010., Strosznajder *et al*., 2010).

The reaction is mediated by mono- and poly(ADP-ribosyl) transferases (PARPs), enzymes that use NAD^+ as substrate to transfer successive ADP-ribose units to glutamic or aspartic acid residues of target proteins, giving rise to long and branched ADP-ribose polymers (Amè *et al*., 2004).

The synthesis of poly-ADP-ribose requires three distinct steps:

- hydrolysis of N-glycosidic bond between nicotinamide and ribose, with release of molecules of adenosine diphosphate ribose (ADPR), and concomitant formation of an ester bond between ADPR and the first unit of a specific amino acid of the acceptor protein.
- Elongation whereby further ADP-ribose moieties are attached to protein bound ADP-ribosyl residues.
- Introduction of an ADP-ribose residue which branches off from a linear portion of the polymer.

All the members belonging to the PARP family have different size, cellular localization and role (Nguewa *et al*., 2005).

The most know PARP-1 and PARP-2, PARP-3, tankirase1, v-PARP are localized in different subcellular sites, respectively nucleus, centrosome, telomers and vaults particle in cytoplasm (Hassa *et al*., 2008). Some of the recently discovered PARPs (PARP-6, PARP-16 and PARP-10) seem to be closer to ADP-rybosyltransferases, as they catalyze mono-ADP-ribosylation, (Kleine *et al.,* 2008).

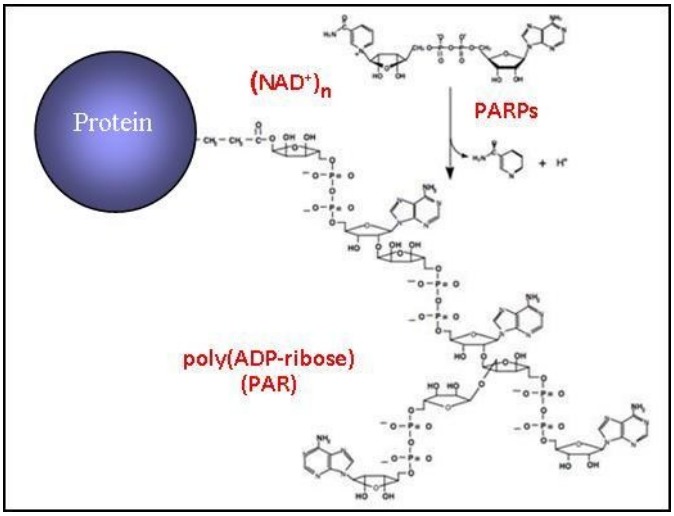

Figure 1: Chemical structures of Nicotinamide Adenine Dinucleotide (NAD^+) and poly-ADP-ribose (PAR). The synthesis of long and branched PAR, catalyzed by PARPs is described.

Hottiger *et al.,* (2010), proposed a new nomenclature based on sequence and structure homology search of the conservative ADP-ribosyltransferase fold and enlarged the family of ADP-ribosylating enzymes by including mono- and poly- ADP-ribosylation proteins, and named all members as ARTs.

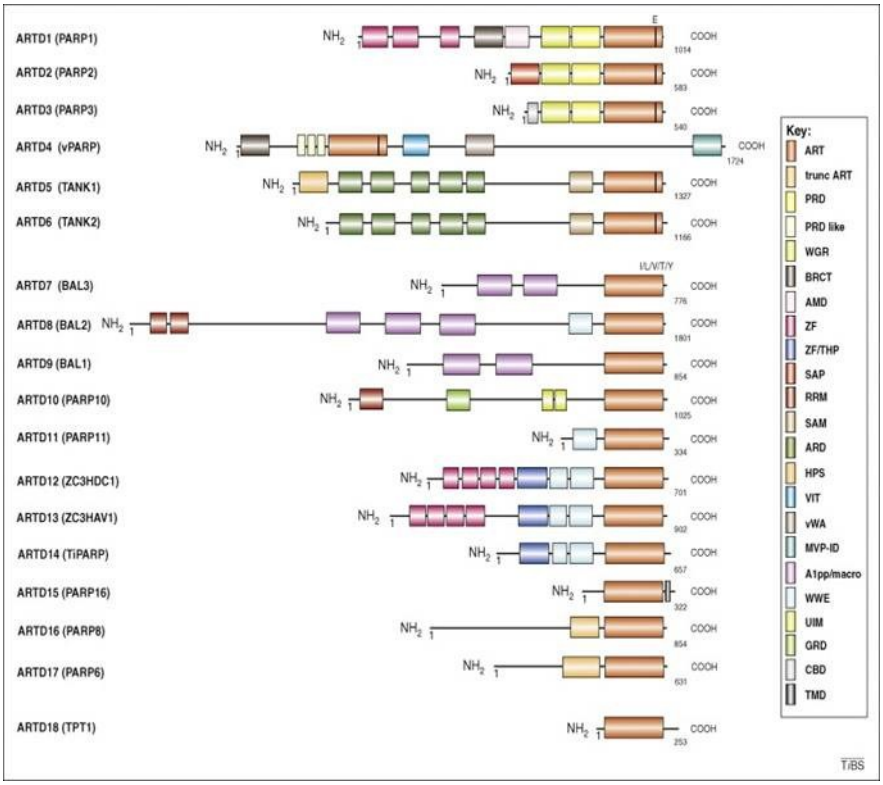

Figure 2: A new schematic comparison of the functional domains of the human ARTD (PARP) family.
The most relevant are: **ART,** is the catalytic core required for basal ART activity. PARP regulatory domain (**PRD**) might be involved in regulation of the PARP-branching activity. **WGR,** characterized by the conserved central motif (W-G-R), is also found in a variety of a polyA polymerase and in proteins of unknow function. **BRCT** domain (BRCA 1 carboxy-terminaldomain) is found within many DNA damage repair and cell cycle checkpoint protein. **Macro** domain can serve as ADPR or 0-acetyl-ADPribose binding module. **ZF:** zinc finger domains, recognizing DNA breaks (Hottiger *et al*., 2010).

Poly(ADP-ribose) polymerase-1 (PARP-1), also known as poly (ADP-ribose) transferase and poly(ADP-ribose) synthetase is the most extensively studied and the founding member of the PARP family. It is a nuclear protein (113kDa) having highly conserved structural and functional organization including an N-terminal double zinc finger DNA-binding domain (DBD), a nuclear localization signal, a central automodification domain (PARP-1 itself is an acceptor of poly-ADPR), and a C-terminal catalytic domain as shown in Figure 3 (Eustermann *et al*., 2011; Tao *et al*., 2009., Virág, *et al*., 2005).

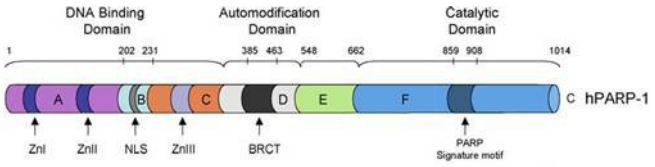

Figure 3: Schematic molecular structure of human PARP-1 enzyme domains (Mègnin-Chanet *et al*., 2010).

DBD of PARP-1 binds to single or double strand breaks with high affinity through its two zinc fingers.

The first zinc finger is essential for PARP-1 activation by DNA double strand breaks, whereas the second zinc finger is important for PARP-1 activation by single strand breaks but not the double strand breaks. When DNA is moderately damaged, PARP-1 participates in DNA repair process and cell survival.

However, in case of extensive DNA damage, PARP-1 over activation induces a decrease in NAD^+ and consequently in ATP levels, leading to cell dysfunction and death (Gagne *et al*., 2006; Meyer-Ficca *et al*., 2005). Therefore over activation of PARP was implicated in the pathogenesis of

several diseases including stroke (Kauppinen *et al*., 2007), myocardial infarction (Szabo *et al*., 2005), neurodegenerative disorders (Koh *et al*., 2005), diabetes (De la Lastra et al., 2007) and several other inflammatory disorders several type of tumors and cellular death (Hamby et al., 2007).

To this regard, PARP-1 is considered as an effective biological marker in many types of human tumours, including melanoma (Mègnin-Chanet *et al*., 2010., Zaremba *et al*., 2009). It's known that many tumors are resistant to classic anti-tumoral drugs which most probably inhibit the apoptotic pathways. In fact, in tumor cells anti-tumor drugs can induce only necrosis, being ATP levels very low to support the apoptotic process.

In this optic, the role of PARP as a molecular switch between necrosis and apoptosis led to investigate about the use of inhibitors of PARP-1, to reduce high consumption of ATP and thereafter to induce apoptosis in tumoral cells. (Fauzee *et al*., 2010., Chol Ha *et al*., 1999).

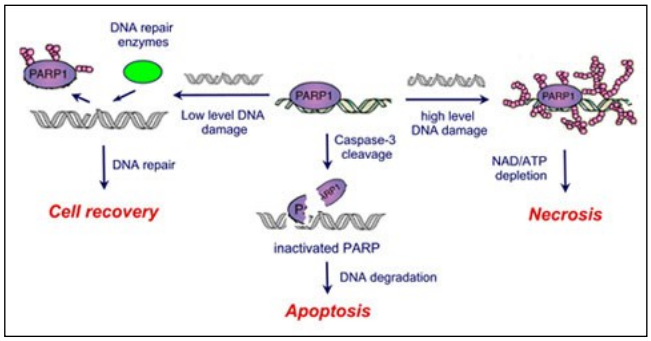

Figure 4: Intensity of DNA damage determines the fate of cell. In case of low levels of DNA damage poly(ADP)ribosilation causes DNA repair and cellular recovery. Moderate levels of DNA damage activates apoptotic pathway. Severe DNA damage induces iperactivation of PARP depleting NAD^+/ATP stores resulting in necrosis (Rupinder *et al.*, 2010).

PARP-1 inhibitors increase antitumoral drugs activity representing a potential new strategy to control haematological and solid malignancies (Comen *et al*., 2010., Peralta *et al*., 2009).

In addition, PARP-1 is the best marker of apoptosis, as in the last phase of this process, caspase 3 and 7 recognize the motif in the nuclear localization signal of PARP-1 and cleave it into two fragments, ~p89 and ~p24; the cleavage at this site separates the DNA binding domain from the catalytic domain rendering PARP inactive.

Recent studies show that PARP-1 plays a role in caspase independent apoptotic cell death by AIF (apoptosis inducing factor); PARP-1 activation induces the translocation of AIF from mitochondria to nucleus, where it causes DNA fragmentation and apoptotic cell death (Koh *et al*., 2005).

Beside its catalytic function, PARP-1 is also partner of other proteins to form complex regulating different nuclear functions and contributing to the maintenance of genomic stability. It has been widely demonstrated the role of PARP-1 as coactivator of Nuclear factor-kB. The automodification of PARP-1 positively up-regulates formation of the NF-kB-DNA complex and enhances transcriptional activation. However, unmodified PARP-1 reduces the DNA binding of NF-kB via its physical association with NF-kB (Chang *et al*., 2001., Hassa *et al*., 2006).

Several studies led to the discovery of a second DNA-damage-activated PARP, PARP-2, that is also a nuclear protein and forms PAR in response to DNA damage. The activity of PARP-2 accounts for approximately 10% of the total PARP activity of human cells (Amè *et al*., 1999). Among the PARP family members, PARP-2 is the closest relative of PARP-1 with their catalytic F-domains having approximately 69% similarity. The N-terminal

domain of murine PARP-2 does not contain zinc-finger motifs but a highly basic DBD, a NLS and a nucleolar localization signal which displays homology with the SAP domain found in various nuclear proteins like APE-1 and Ku-70, involved in chromosomal organization or DNA repair. The PARP-2 DBD is structurally different from that of PARP-1, probably reflecting differences in the DNA structures recognized by each enzyme (Amè *et al*., 2005). The PARP-2 E domain acts both as the interacting interface with various partners and as an auto-modification domain. PARP-1 and PARP-2 can homo-and hetero-dimerize and poly(ADP-ribose)ate each other (Schreiber *et al*., 2004).

1.2 Poly(ADP- ribose) catabolism

The removal of PAR from the modified proteins is catalyzed by an enzyme, the poly(ADP-ribose) glycohydrolase (PARG), which is encoded by a single gene (Min, *et al*., 2009., Gagne *et al*., 2006). PARG possesses an exo- and endoglycosidase activity that hydrolyzes ribosyl-ribose bonds and produces free ADP-ribose (ADPR) residues from both linear and branched poly(ADP-ribose). Thereafter an ADP-ribose lyase is responsible for the cleavage between the first ADP-ribose and modified amino acids. The concerted action of these enzymes modulates the level and complexity of polymer on different acceptor proteins (Bonocalzi *et al*., 2005), preventing an accumulation of highly modified nuclear proteins with very long chains of PAR, including PARP-1 itself. The catabolism of poly-(ADPR) allows PARP-1 to remain active in conditions of DNA damage, because it assures interactions between the enzyme and genoma.

It has been demonstrated that the gene encoding human PARG is alternatively spliced to generate three different protein isoforms with molecular weights of, 110, 103 and 99 kDa, respectively (Meyer-Ficca *et al*., 2004). Several studies have shown that PARG110 localizes predominantly to the nucleus (Ohashi *et al*., 2003), while PARG103 and PARG99 isoforms are cytoplasmic (Bonocalzi *et al*., 2003). Interestingly, the cytoplasmic PARG103 isoform accounts for nearly 90% of the entire cellular activity . The PARG enzyme contains a C-terminal catalytic domain and a N-terminal putative regulatory domain which would be involved in protein–protein interactions (Ame *et al*., 1999).

Importantly, all PARG isoforms, like PARP-1, has been found to be efficiently cleaved by caspase-3 in human cells undergoing apoptosis (Affar *et al*., 2001). This cleavage of PARG releases enzymatically active C-terminal fragments of 85 or 74kDa and these PARG85 and PARG74 apoptotic fragments are localized in the cytoplasm too (Bonocalzi *et al*., 2003). Apart from these observations, little is known about the biological significance of the cellular localization and the presence of different PARG isoforms other than they are able to catalyze PAR turnover in different cell compartments. The basal levels of polymer within unstimulated cells are usually very low (Andrabi *et al*., 2006) and the nuclear concentration in mammalian cells is within the range of one to two times the Km value of PARG (Alvarez-Gonzalez, *et al*., 1989).

Therefore, the nuclear PAR concentration is sufficient to maintain a constant PARG activity, even in unstimulated cells. In response to genotoxic stress, the levels of PAR can increase by 10- to 500-fold and this event leads to caspase independent cell death (Kang *et al*., 2004., Yu *et al*., 2003) supporting the hypothesis that PAR turnover is essential for cell survival.

Thus, it is extremely important that both cytoplasmic and nuclear levels of ADP-ribose polymers are tightly controlled in order to prevent PARP-1 induced cell death (Koh, *et al*., 2005., Masutani *et al*., 2003). Therefore, it is not surprising that PARG activity has been found to be critical for the prevention of PARP-1 dependent cell death by regulating the intracellular levels of PAR (Ying *et al*., 2001).

1.3 Melanoma

Melanoma is the most aggressive form of skin cancer. It originates from neoplastic transformation of melanocyte cells, involved in the production of melanin, the dark pigment that is responsible for giving skin its colour. They predominantly occur in skin, but are also found in other parts of the body, including the bowel and the eye. Its incidence has increased in the last decade mainly among the young people.

Although causality has been difficult to link with melanoma, sun exposure and genetic susceptibility are considered important predisposing factors (Amiri *et al*., 2005).

The development and progression is viewed in five distinct stages. The common acquired nevus is the first step followed by dysplastic nevus showing an increased level of structural and architectural atypia.

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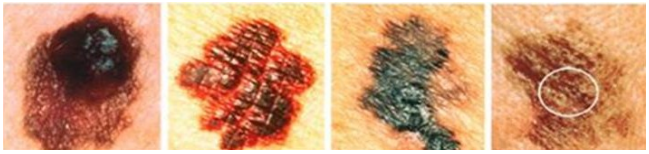

Figure 5: Morphological changes in the onset of melanoma.

The third step is the first recognizable malignant stage, defined as the radial growth phase (RGP) primary melanoma. The cells in this phase are locally invasive but they lack metastatic capacity. Radial growth phase cells can progress to vertical growth phase (VGP) primary melanoma lesions, the fourth step in progression.

In this step, melanoma cells infiltrate and invade the dermis as large clusters of cells and exhibit metastatic potential. Metastasis to distant organs followed by overgrowth of tumor cells at these sites is the final step in the progression process (Clark *et al*., 1991).

Although this model depicts melanoma progression as a result of series of genetic defects intrinsic to melanocytes, our current knowledges about the importance of the tumor microenvironment in control of growth, differentiation, invasion and metastasis adds to the complexity of the disease, hence the challenges faced both in clinics and research laboratories in the fight against melanoma.

Due to the complex nature of the disease, melanoma has proven to be highly resistant to conventional chemotherapy with dacarbazine (DTIC) or its derivative Temozolomide (TMZ) having the best single agent activity with a response rate of only 15–20% and a short 4 month median response duration (Sun *et al*., 2001). Patients at high risk for recurrence (stage III) are frequently given IFN-α as adjuvant treatment, although its effectiveness is considered low as compared to toxicity (Punt *et al*., 2001). Patients with metastatic disease (stage IV) have a median survival of 6–10 months with a 5-year survival of <5% (Balch *et al*., 2001).

Recent advances to understand the underlying biology in progression of melanoma have identified key signaling pathways that are important in promoting melanoma tumorigenesis, thus providing dynamic targets for therapy. One such important target identified in melanoma tumor progression is the nuclear factor-kappa B (NF-κB) pathway (Lin *et al*., 2011).

1.4 NF-kB and NBD-peptide

NF-kB is an inducible transcription factor involved in normal immune response which can be activated by different stimuli, including pro-inflammatory cytokines, "non-self" antigens, lymphocyte mitogens, and cellular stress (ultraviolet and ionizing radiations, as well as chemotherapeutic agents).

Constitutive activation of NF-*κ*B is an emerging hallmark of various types of tumors and many experimental models *in vitro* and in animals indicate the role of this transcription factor in the regulation of apoptosis, tumor angiogenesis and proliferation, as well as tumor cell invasion and metastasis. (Karin *et al*., 2006., Ueda *et al*., 2006).

The enhanced activation of NF-kB in tumors appears to be partially due to deregulation of upstream kinases such as Ras, Raf, NIK, and AKT that impinge on the NF-kB pathway.

The mammalian NF-kB family contains five proteins, RelA/p65, NF-kB1 (p50), NF-kB2 (p52), c-Rel and RelB. They exist as homodimers or heterodimers with a wide range of DNA binding and activation potentials. In most unstimulated cells NF-kB is sequestered in the citoplasm as an inactive transcription factor in a complex with one of inhibitors of NF-kB (IkB alfa, beta).

In the presence of a wide range of stimuli, the inhibitors are phosphorylated by IkB kinase (IKK) complex and degradated by 26S proteasomes (Baldwin *et al*., 2001). Upon activation, NF-kB dimers enter the nucleus, where they modulate transcription of many genes encoding cytokines, growth factors, cell adhesion molecules, and antiapoptotic proteins (Yang *et al*., 2007).

The IKK complex is composed of three subunits: the catalytic subunits IKK-α and IKK-β, and the regulatory subunit IKK-γ (also known as NEMO). NEMO regulates the IKK complex activity through its binding to carboxyl-terminal region of IKKα and IKKβ, termed NEMO-binding domain (NBD) (May *et al*., 2002).

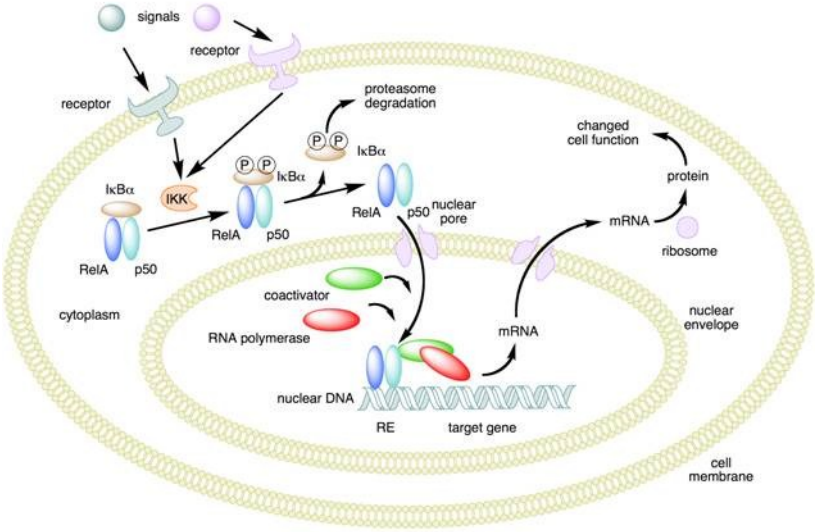

Figure 6: NF-kB pathways. Signalling network leading to NF-kB activation (see text for description).

Recently, a cell-permeable NBD-peptide has been shown to block the association of NEMO with the IKK complex, inhibiting NF-kB activation and ameliorating inflammatory responses. This peptide contains carboxyl-terminal region of IKK complex catalytic subunits fused with Penetratin, a *Drosophila melanogaster* protein that crosses cell membranes and facilitates the entry of the peptide in the cytosol. Once inside, the peptide prevents the assembly of the catalytic subunit NEMO, thereby destroying the structural and functional integrity of the IKK complex (Di Meglio *et al*., 2005).

The potential of this peptide as an anti-inflammatory agent has been demonstrated in vivo in various animal models. Importantly, the NBD peptide does not completely inhibit NF-kB activity, suggesting that selective disruption of the interaction of NEMO and IKK will most likely leave residual NF-KB activity that might be sufficient to maintain normal cellular processes.

1.5 Scientific hypothesis and aim of the work

Recently, numerous studies have demonstrated that NF-kB is connected with multiple aspects of oncogenesis. In this perspective, inhibition of NF-kB is expected to be an ideal therapeutic target in those tumours where NF-kB appears to play a unique survival role. In vitro experiments have shown that NF-kB activity is constitutively elevated in human melanoma cultures compared to normal melanocytes.

The identification of the NEMO-binding domain (NBD) peptide, able to block the activation of the IKK complex has provided the possibility to selectively modulate the activation of NF-kB transcriptional nuclear factor by targeting the NBD-NEMO interaction.

In this, aim of present research was to verify whether NBD-peptide is able to induce programmed cell death in A375 melanoma cells. For this purpose, in the first part of the research, we studied cell proliferation at different NBD-peptide concentration and NF-kB activity. Later, the apoptosis induction was analysed by flow citometry and expression of PARP-1, that is a known marker of this process.

In the second part of this work, the poly-ADP-ribosylating system has been characterized both in melanocytes and untreated and treated A375 cells. In this reaction products of PARP1 have been isolated from the treated and untreated cells and used as substrate to determine PARG activity.

Therefore, further seven melanoma cell lines with different mutations were examined to verify and/or confirm the apoptosis induction following NBD-peptide treatment.

MATERIALS AND METHODS

2.1 Cell lines

Melanocytes are melanin-producing cells located in the bottom layer (the stratum basale) of the skin's epidermis. They have an elongated form, a large nucleus and grow in clusters (Figure 7).

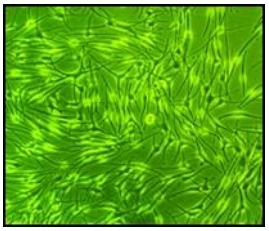

Figure 7: Melanocyte cells.

The A375 melanoma cells are highly invasive and spontaneously metastatic. Morphologically they have undifferentiated shape aspect (form). They have a lengthened or polygonal form depending on whether they are plated or in suspension (Figure 8A). Moreover, these cells grow through cell-cell interaction and in multilayer, so they shape polyhedric and form oval colony (Figure 8B). The A375 are changed in BRAF (Serine/threonine protein kinase) and therefore the MAPK pathway is constitutively activated.

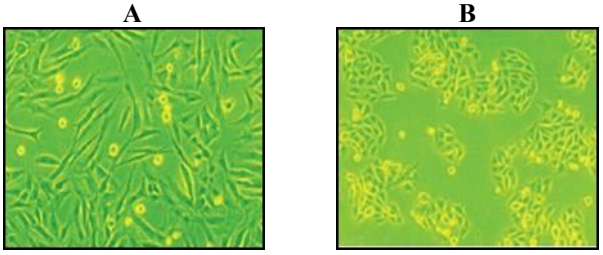

Figure 8: A375 melanoma cells (A) and (B).

2.2 Cell growth and treatment with NBD- peptide

A375 melanoma cell line and normal human epidermal melanocytes (NHEM) were purchased from Promo Cell (Heidelberg, D-69126, Germany). Melanocytes were grown in medium 2 (Promo Cell). A375 in complete Dulbecco's modified Eagle's medium (DMEM). The media were supplemented with 10% FBS, 2mM L-glutamine, additioned with antibiotics (100U/ml penicillin, 100 mg/ml streptomycin) and cells maintained in humidified atmosphere with 5% CO_2 at 37°C. Cell count was performed at 0, 24, 48, 72 and 96 hours (Figure 9).

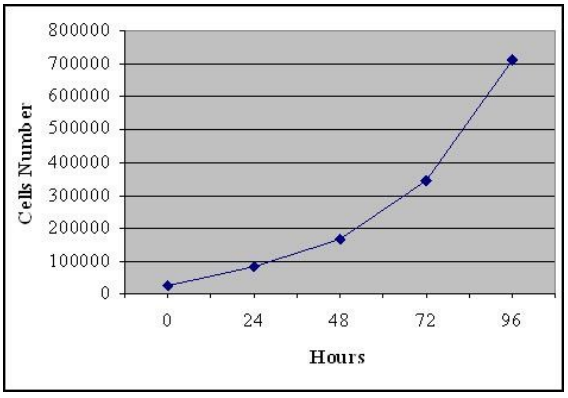

Figure 9: Growth curve of A375 cell line.
The curve was obtained by counting cells at 0, 24, 48, 72 and 96 hours. These cells show a short replication cycle, in which growth is influenced by density of plating.

To investigate whether treatment with NBD-peptide would lead to cell death, cell survival was measured by trypan blue dye esclusion on A375 cell line; NHEM was used as control. The cells were plated at the concentration of 1 x 10^5 cells/well and were cultured in 2 mL DMEM complete medium with NBD-peptide from Genosphere Biotech (Paris, France) at 6.25, 12.5,

25, 50, 100μM. NBD-peptide was dissolved in DMSO and stock solution was stored at -20°C. After incubation for 24 h, cells were collected and an aliquot of cell suspension was mixed with equal volume of trypan blue 0.4%. Viability was determined at the designed intervals by vital-dye exclusion assay. The same experiment was carried out at 50 and 100μM peptide for 3 hours.

2.3 Electrophoretic mobility shift assay (EMSA)

The cells treated with NBD-peptide (50μM), were prepared after lysis in extraction buffer (50mM Tris [tris(hydroxymethyl)aminomethane]-HCI, pH 7.4, 150mM NaCI, 0.1%Triton X-l00, 5mM EDTA [ethylenediaminetetraacetic acid], 1mM Na3V04, and 1mM PMSF [phenylmethylsulfonyl fluoride] and protease inhibitors. Double-stranded containing the NF-kB (5'-CAACGGCAGGGGAATCTCCCTCTCCT-3T') recognition sequence were endlabelled with [^{32}P] -γ-*ATP*.

Aliquots of whole-A375 cell extracts (12 μg protein/sample), treated for different time with NBD-peptide were incubated with [^{32}P] -labelled kB-DNA probe in binding buffer for 30 min (Piva *et al*., 2005). DNA-protein complexes were analyzed by non-denaturing 4% polyacrylamide gel electrophoresis in 1x TBE buffer at 150 V for 2 hours at 4°C. The gel was dried and autoradiographed using an intensifying screen at -80°C for 20 hours.

NF-kB-DNA complex formation was quantitatively determined by densitometric analysis performed with a GS-700 imaging densitometer (Bio-Rad, Italy) and a data computer program (Molecular Analyst; IBM).

2.4 Kinase assay

Cell lysates were immunoprecipitated with anti-IKKa antibodies (Cell Signaling Technology) in the presence of 20μL protein-A-Sepharose (Sigma) at 4°C for 16 hours. After extensive washing, endogenous IKK activity was determined using glutathione-Stransferase (GST)-IκBα as substrate (Piva *et al*., 2005).

2.5 Flow cytometry

To evaluate apoptosis after NBD treatment, cells were treated for 3 hours with 6.25, 12.5, 25, 50μM of NBD peptide. Cells were detached by Trypsin/EDTA and apoptosis was detected by measuring the active form of caspase-3 by FACS Vantage Cell Sorter cytometer (BD Biosciences, San Diego,CA) using the PE-conjugated anti-human-active caspase-3 monoclonal antibody (mAb) (BD Biosciences, San Diego, CA) and following the manufacturer's instructions. The Data obtained were analyzed by Cell Quest Software. Mononuclear cells were washed, fixed, and permeabilized by the Cytofix/Cytoperm™ Kit (BD Biosciences, San Diego,CA). Briefly, cells were pelleted for 20 min at room temperature (RT), and washed with Perm/Wash™ Buffer C. Cells were then stained with anti-active caspase-3 mAb using 10 μl / 1x 10^5 cells for 60 min at RT in the dark, washed with Perm/Wash™ Buffer, resuspended in PBS and analyzed by flow cytometry.

2.6 Cell lysis and isolation of cell nuclei

Nuclear fractions from melanocytes (5 x 10^5 cells), melanoma control cells (5 x 10^5 cells), and melanoma cells (5 x 10^5 cells), treated with NBD-peptide were isolated by Quiagen kit. Whole fractions were used for the following biochemical analyses.

2.7 Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulphate, and Western Blotting

Nuclear fractions (20μg of proteins) from melanocytes, treated and untreated A375 were analyzed on 12% polyacrylamide gels in the presence of 0.1% SDS according to (Nicholas *et al*., 1982). Gels were stained in 0.1% Coomassie G in 10% acetic acid 30% methanol. For immunoblotting electrophoresed (20μg) proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Biorad) at 200 V for 1.5 h at 4°C in the same buffer used for electophoretic run.

For immunoblot experiments procedures and buffers were according to (Faraone Mennella *et al*., 2005). PVDF sheets were treated for 1.5 h with blocking solution (50mM Tris-HCl buffer, pH 8.0; 150mM NaCl; 0.5% (v/v), Tween 20 and 3% gelatine). Incubation with commercial anti- PARP 1/2 (Santa Cruz, rabbit anti-human PARP, H-250,1:2000, v/v), was performed for 2 h at room temperature in the same solution supplemented with 0.3% gelatine.

The filters were washed several times with TBS-Tween and antibody binding was detected by Anti-rabbit IgG (H+L) horseradish peroxidase (1:2000;v/v). Their action was revealed by using a kit for

chemiluminescence (Super signal West Dura, Extended Substrate, Pierce) and reading by a Quantitaty One program in a Chemidoc apparatus (Bio-Rad).

2.8 PARP activity

The ADP-ribosylating activity of the PARP enzyme in whole nuclear fractions (20µg proteins) from melanocytes, untreated and treated melanoma cells was routinely assayed for 10 minutes at 25°C in the presence of 0.51mM [^{32}P]NAD^+ (GE Healthcare Europe GmbH, Biosciences) (10, 000 cpm/nmole) (Faraone Mennella *et al*., 2003). The final specific radioactivity (10,000 cpm/nmole) and concentration (0.51mM) were reached by mixing labelled and unlabelled 0.51mM NAD^+. The reaction mixture was composed of 500mM Tris-HCl buffer, pH 8.0; 50mM $MgCl_2$ and 10mM DTT (final volume 50µl).The reaction was stopped with ice-cold 30% trichloroacetic acid (TCA) and the radioactivity present in the acid insoluble material, collected on HAWP filter (0.45µm, Millipore), determined on a Beckman LS 1701 liquid scintillation spectrometer.One PARP milliunit (mU) is defined as the amount of enzyme required to converte 1nmol of NAD^+/min. under standard conditions.

2.9 Covalent ADPR Protein Acceptors

Nuclear fractions (20µg proteins) from melanocytes and untreated and treated (50µM peptide for 6h) cells were incubated under standard conditions with [^{32}P]NAD^+ (50,000 cpm/nmole). The reaction was

stopped by transferring the mixture on dry ice and liophylizing. The dried samples were suspended in electophoretic buffer and loaded on polyacrylamide (12%) gel. Images of stained gels and autoradiographic patterns of labeled proteins were acquired by a Phosphor imager (mod. Fx, Biorad). Densitometric analysis of radioactive ADPR protein acceptors was performed using Personal molecular image FX and Quantity One Programm (BIO-RAD).

2.10 Western blotting with anti-PARG antibodies

Nuclear extracts (20μg proteins) from melanocytes, untreated and treated A375 melanoma cells (NBD peptide 50μM for 6h) were separated by SDS-PAGE. Western Blot analysis was performed as previously described, using anti-PARG MaxPab mouse polyclonal (B01) (Abova, 1:200; v/v) and anti-mouse IgG (H+L) horseradish peroxidase (Pierce, 1:1000; v/v), as primary and secondary antibodies respectively.

2.11 PARG purification

Poly(ADP-ribose) glycohydrolase was purified from untreated and treated A375 by electroeluition of band corresponding to molecular weight of PARG enzyme from SDS–PAGE 12%.

After electrophoresis of nuclear fractions (200μg proteins), the gel was stained for 15 min with Coomassie Blue G. As soon as the protein of interest became visible, the band was cut out with a razor blade. The gel piece was washed and placed into a dialysis tube (14, 000 c.o.), containing

1mL of electroelution buffer (0.01M Tris-acetate pH 8.6). The tube was placed in the electroelution apparatus containing 0.01M Tris-acetate pH 8.6 in the central lodging and 0.04M Tris-acetate in the lateral lodging. The electroelution was carried out at 8mA overnight. The concentration of electroeluted protein was determined by the Bradford method (Bradford *et al*., 1976). The electroeluted protein was analyzed by SDS-PAGE and identified by Western Blotting with anti-PARG antibodies as already described.

2.12 PolyADP-ribosylating proteins determination after PARG assay

Two nuclear fractions (each of 100μg) from untreated and treated A375 cells were incubated with 0.51mM [^{32}P] NAD^+ (10,000 cpm/nmole) in a reaction mixture composed of 500mM Tris-HCl buffer, pH 8.0; 50mM $MgCl_2$ and 10mM DTT (final volume 50μl), according to PARP standard assay described in (De Lucia *et al*., 1996).

The reaction was stopped with ice and the ADP-ribosylating proteins were precipitated by trichloroacetic acid (TCA) (30% final) and washed with ethanol. One of two fractions from each samples, containing the ADP-ribosylating proteins were re-incubated in a 50μl reaction mixture containing 100mM Tris-HCl, pH 8.0, 10mM dithiothreitol and purified PARG (6μg/ml) (PARG assay). The other fractions were incubated in the same reaction mixtures without PARG enzyme (control). The reactions were stopped by addition of ice-cold TCA (30% final) and the residual radioactivity associated to proteins was collected on HAWP filter (0.45μm,

Millipore) and measured by a Beckman LS 1701 liquid scintillation spectrometer.

2.13 Synthesis and purification of protein-free poly(ADP-ribose)

Protein-free [^{32}P] Poly(ADP-ribose) from nuclear fractions untreated and treated (50μM peptide) cells were synthesized in a 500μl reaction mixture containing 500mM Tris-HCl buffer, pH 8.0; 50mM $MgCl_2$, 10mM DTT and 0.51mM [^{32}P] NAD (100,000 cpm /nmol). The reaction mixture was incubated for 10 min at 25°C and proteins precipitated by 20% trichloroacetic acid. Subsequently, this pellet was incubated with Tris NaOH 10mM pH 12, containing 1mM EDTA for 2 h at 60°C to release polymers from protein. Finally, protein-free [^{32}P]poly(ADP-ribose) was extracted three times with isoamilic alcohol/chloroform (1:49; v/v) and separated by 20% polyacrylamide gel electrophoresis, according to (Panzeter *et al*., 1993). Finally, dried gel was exposed to autoradiography film over night and revealed by Phosphor imager (mod. Fx, BIO-RAD).

2.14. Electrophoretic identification of PARG reaction products

[^{32}P]poly(ADP-ribose) (2,000 cpm, 70 nCi/nmol ADP-ribose) and purified PARG (6μg/ml) [^{32}P]poly(ADP-ribose) were incubated for 5, 10 and 15 minutes at 37°C in a 50 μl reaction mixture containing 100mM Tris-HCl, pH 8.0, 10mM dithiothreitol (standard PARG assay). The reaction was stopped by addition of SDS 01% final concentration and transferring the mixtures to 60°C for 10 min. The digested products of [^{32}P]poly(ADP-ribose) were freed of proteins by incubation with Proteinase K (SIGMA,

0.2 mg/ml) for 1 h at 37°C and separated by 20% polyacrylamide gel electrophoresis (Panzeter *et al*., 1993).

The dried gel was exposed to autoradiography film over night and revealed by Phosphor imager (mod. Fx, BIO-RAD).

2.15 Different melanoma cell lines

SK-MEL2, 397, SK-MEL5 , M14, LOX , SK-MEL28, UACC257 were from NCI-Frederick Cancer Center DCTD Tumor/Cell Repository were cultured in RPMI1640 (Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 10% FBS (Htclone, Logan, UT, USA), 2mM L-glutamine, additioned with antibiotics (100U/ml penicillin, 100 mg/ml streptomycin) and maintained in humidified atmosphere with 5% CO_2 at 37°C.

The cells treated with NBD-peptide at the concentration of 50μM for 6 hours, were analyzed by flow cytometry and western blotting with anti-PARP antibodies as previously described for A375 cells. PARP activity of whole cells was also determined.

RESULTS

3.1 Treatment of A375 cells by different concentrations of NBD-peptide

A375 melanoma cells were treated with NBD-peptide at concentrations of 6µM, 12µM, 25µM, 50µM and 100µM for 24h (Figure 10). This treatment induces a dose-dependent inhibition of melanoma proliferation. The highest inhibition of proliferation (60%) occurs when A375 cells are treated with 50µM peptide. A higher concentration of peptide (100µM) does not produce further inhibition after 24 hours of incubation (Figure 10). Comparable results were obtained in the presence of 50µM peptide for 3h (data not shown).

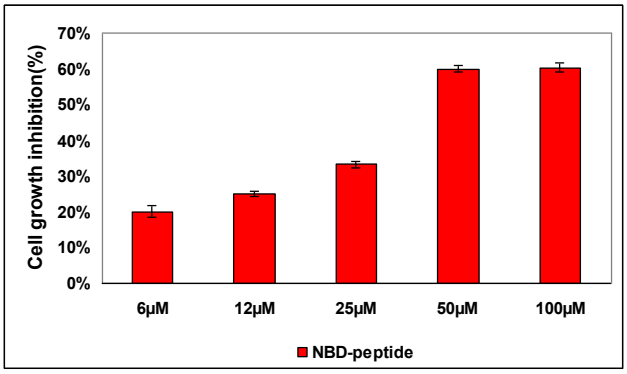

Figure 10: Inhibition of A375 cell growth by NBD peptide treatment for 24 hours. Different concentrations of NBD-peptide were tested, with maximal inhibition at 50µM of peptide. As negative control melanocytes growth was tested also in presence of 50 µM NBD-peptide (data not shown).

3.2 Modulation by NBD-peptide of NF-kB activation

The experiment was focused to test both the levels of NF-kB activity by EMSA assay, and to study the role of IKK complex and its regulation by NBD-peptide. First, cells were treated with 50μM NBD-peptide to get maximal growth inhibition for different time. Samples were analyzed by EMSA assay, in order to investigate whether the peptide modulates NF-kB activity. EMSA assay was carried out using the NF-kB (5'-CAACGGCAGGGGAATCTCCCTCTCCT-3T') recognition sequence, end-labelled with [^{32}P]-γ-ATP (as described in Materials and Methods, 2.3). The autoradiography of electrophoretic mobility shift assay showed a reduction of NF-kB activity, in a time-dependent manner, as confirmed by densitometric analysis (Figure 11A and B).

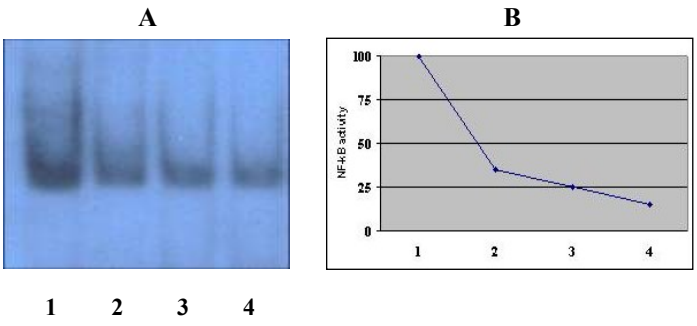

Figure 11: Modulation of NF-kB activity in A375 cells treated with 50 μM of NBD-peptide for 3h, 6h, and 24h.
(A) Non-denaturating 4% polyacrylamide gel electrophoresis; (B) Densitometric Analysis. 1= untreated A375 cells; 2= A375 cells treated with (50μM) NBD- peptide for 3h; 3= A375 cells treated with (50μM) NBD-peptide for 6h; 4= A375 cells treated with (50μM) NBD-peptide for 24h.

On the other hand to determine whether inhibition of constitutive NF-kB activity could be the result of down regulation of the upstream IKK activity by the NBD-peptide, we performed an IKK-activity assay on A375 human melanoma cells.

The high activity of IKK in A375 human melanoma cells, measured as incorporation of radiolabelled [^{32}P] into GST-IKBα, was reduced by the NBD-peptide in a time-dependent fashion. As indicated in Figure 12, the IKK activities were reduced by 46%, 51%, and 66% in response to the NBD-peptide treatment of 50μM for 3, 6 and 24 hours, respectively.

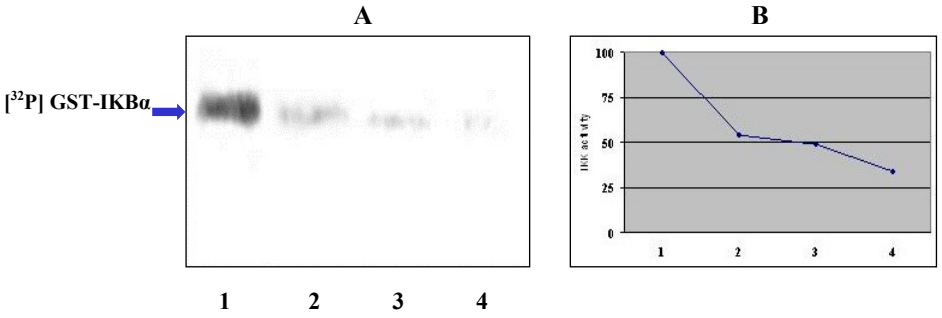

Figure 12: Analysis of reaction products from IKK activity on GST-IKBα by SDS-polyacrylamide gel (10%).
(A) Autoradiography of gel; (B) Densitometric Analysis; 1= untreated A375 cells; 2= A375 cells treated with (50μM) NBD-peptide for 3h; 3= A375 cells treated with (50μM) NBD-peptide for 6h; 4= A375 cells treated with (50μM) NBD- peptide for 24h.

3.3 Effect of NBD-peptide on apoptosis induction

Caspase3 activation and levels of apoptotic PARP-1 fragment (89kDa) are routinely used as marker of apoptosis. Therefore, in order to characterize the mechanism underlying the results described in the previous section, A375 melanoma cells were exposed to the NBD-peptide at different concentrations (6.25, 12.5, 25, and 50μM) for 3h and then were analyzed by flow cytometry after incubation with PE-conjugated anti-human-active caspase-3 monoclonal antibodies (mAb). Results clearly showed a dose-dependent activation (20.3%, 25.0%, 30.9% and 40.2% respectively) (Figure 13). Again the maximal value (40,2%) corresponded to cells treated with 50μM of NBD-peptide.

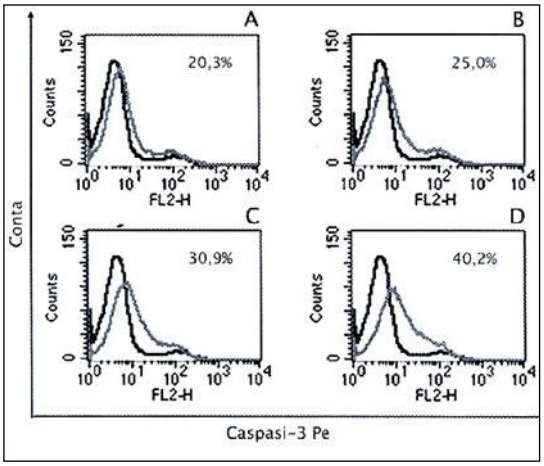

Figure 13: Cytometry analysis of treated A375 NBD-peptide for 3h.
A= 6.25μM NBD-peptide; B= 12.5μM NBD-peptide; C= 25μM NBD-peptide; D= 50μM NBD-peptide. A375 cells were analyzed with the monoclonal antibodies (mAb) PE-conjugated anti-human-active-caspase3.

During apoptosis, PARP-1, the best marker of this process, is cleaved by caspase3 in two fragments of 89kDa and 24kDa, respectively. For this reason, to evidence whether NBD-peptide treatment induces apoptotic process in A375 cells, electrophoretic analysis (Figure 14A) following by western blotting with commercial anti-PARP antibodies (Figure 14B) was carried out on nuclear fractions from melanocytes (control), untreated and treated A375 cells. They were treated with 50μM NBD-peptide for 3, 5, 6 hours.

Immunoblot showed immunoreactive signals of 113kDa (native enzyme) and 89kDa (cleaved form). In contrast to untreated cells, the 113kDa band decreases and the 89kDa increases after NBD treatment. The increase of 89kDa fragment confirms that most A375 cells undergo apoptosis at levels comparable to those of melanocytes.

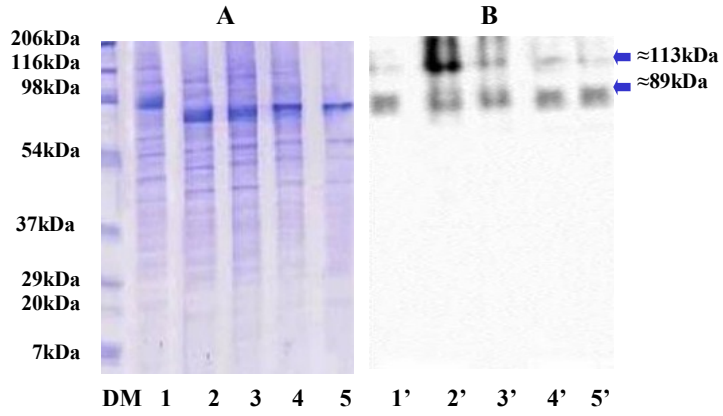

Figure 14: Analysis of nuclear fractions from melanocytes, untreated and treated A375 cells with 50μM of NBD-peptide for 3, 5, 6 hours.
(A) SDS-polyacrylamide gel (12%) stained in Coomassie, (B) Immunoblotting with anti-PARP-1 Abs. Nuclear fractions from: 1=1' melanocytes; 2=2' A375 cells; 3=3' A375 treated with 50μM NBD-peptide for 3h; 4=4' A375 treated with 50μM NBD-peptide for 5h; 5=5' A375 treated with 50μM NBD-peptide for 6h.

3.4 Effect of NBD-peptide on PARP activity.

To confirm the PARP-1 catalytic fragment (89kDa) is inactive, the enzyme activity was measured in nuclear fractions of melanocytes, melanoma cells both before and after treatment with NBD-peptide at 25μM (Figure 15A), 50μM (Figure 15B) and 100μM (Figure 15C) for 3, 5 and 6 hours. Reaction mixtures prepared as described in Materials and Methods (section 2.8) cointained an isotopic dilution of PARP-1 substrate [^{32}P] NAD^+ and the same amount of proteins (20μg). The levels of PARP-1 activity in melanocytes are significantly lower than those measured in A375 melanoma cells (Figure 15). The treatment with NBD-peptide (25 and 50μM) reduces enzyme activity in a time- and a dose-dependent manner. The maximum decrease of enzyme activity (84%) occurs after incubation of tumoral cells with 50 μM peptide for 6 hours (Figure 15B). Further activity reduction is not evident after treatment at higher concentrations of peptide (100μM) (Figure 15C).

As PARP-1 is the best consumer of intracellular energy, its partial inactivation ensures both induction and maintenance of apoptotic state.

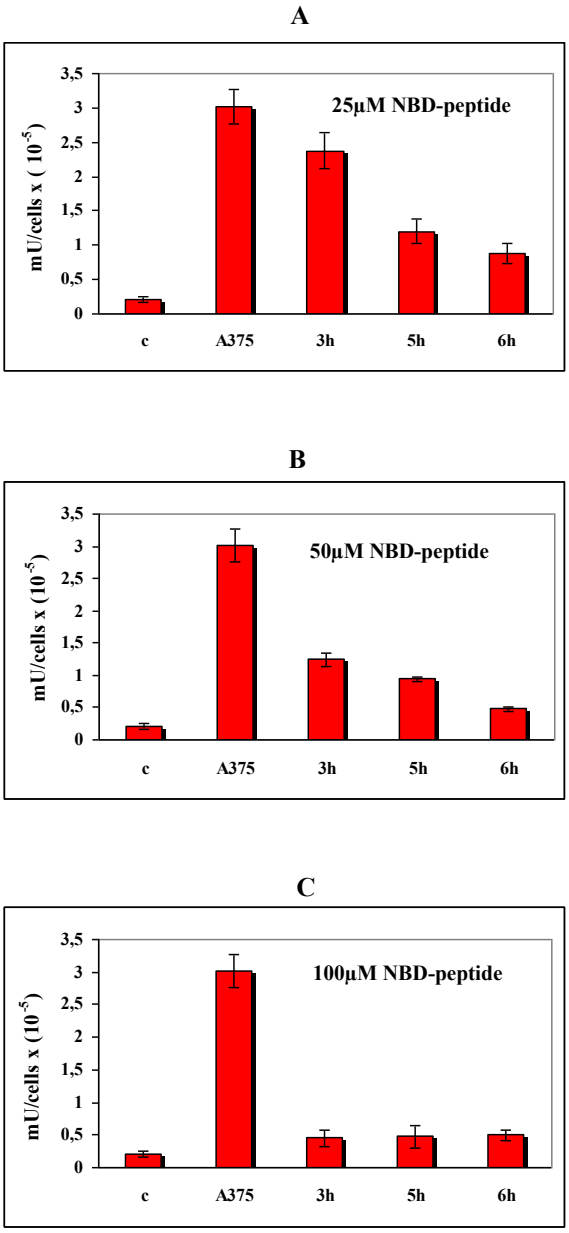

Figure 15: PARP-1 activity in nuclear fractions from melanocytes, untreated and treated A375 cells with 25µM, 50µM and 100µM NBD-peptide.
c= melanocytes; A375= untreated cells; 3h= A375 cells treated for 3h; 5h= A375 cells treated for 5h; 6h= A375 cells treated for 6h.

The same nuclear suspensions of melanocytes, untreated A375 cells and treated cells with 50μM of NBD-peptide for 6h were incubated with [^{32}P] NAD^+ in standard assay conditions and electrophoresed, to evidence covalent protein acceptors of ADPR. The autoradiographic analysis revealed the presence of a single signal to the top of gel (Figure 16A). The densitometric analysis of autoradiographic bands showed that the entity of the modification is bigger in untreated than in treated cells and in melanocytes (Figure 16C). Although, this experiment didn't allow to highlight the possible ADPR acceptors, however it confirmed that the poly-ADPR produced in treated cells decreases following treatment with NBD-peptide. In melanocytes, where the lowest PARP-1 activity was measured, we observed the lowest production of polymer.

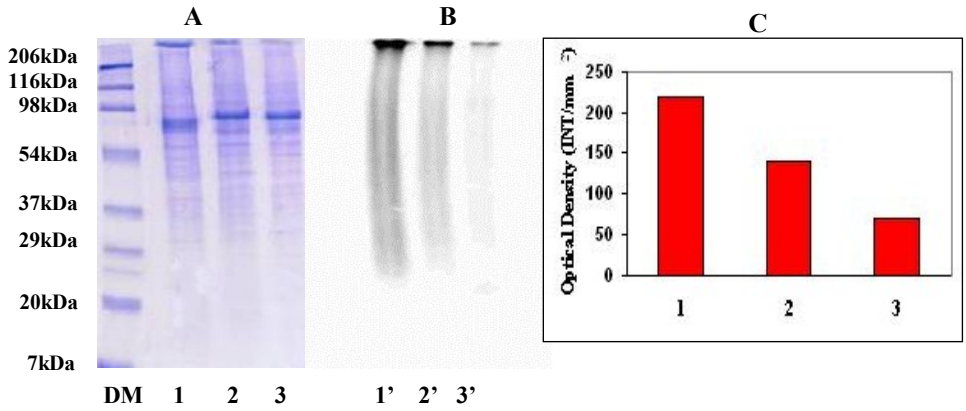

Figure 16: Analysis of protein acceptors.
(A) SDS-polyacrylamide gel (12%) stained in Coomassie, (B) Autoradiographic analysis of protein acceptors and (C) densitometric analysis of B. Nuclear fractions from: 1=1' A375 cells; 2=2' A375 cells treated with 50μM NBD-peptide for 6h; 3=3' melanocytes.

3.5 PolyADP-ribose turnover

The effect of cells treatment with NBD-peptide on PAR turnover was studied. PARG is responsible of the conversion of PAR into ADP-ribose units; therefore its behaviour was analyzed and compared to that of the enzyme of PAR synthesis, PARP-1.

PARG expression in nuclear fractions from untreated and treated A375 cells was evidenced by electophoretic analysis (Figure 17A) followed by Western Blotting with anti-PARG antibodies (Figure 17B). In both samples, a single immunoreactive band corresponding to the PARG isoform with a molecular weight of about 70-75kDa was observed.

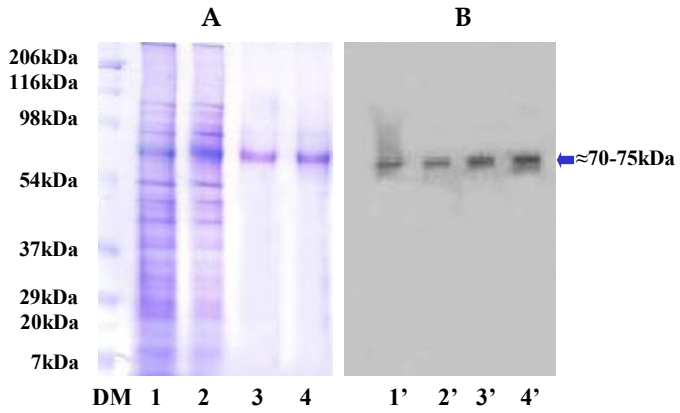

Figure 17: Analysis of PARG enzyme expression from A375 melanoma cells. Treatment was with 50µM NBD-peptide for 6h. (A) SDS-polyacrylamide gel (12%) stained in Coomassie, (B) Immunoblotting with anti-PARG Abs. Nuclear fractions from: 1=1' untreated A375 cells; 2=2' treated cells. 3=3' purified PARG from nuclear fraction of A375 cells; 4= 4' purified PARG from nuclear fraction of treated A375 cells.

Purified PARG fractions from both untreated and treated A375 cells were used to determine enzyme activity at different incubation times (Figure 18). The poly-ADPR produced by PARP assay and protein-bound (acid-insoluble fractions), was used as substrate. The highest levels of [^{32}P]-PAR labelled ADP-ribosylating proteins (taken as 100%) were measured in melanoma A375, as compared with treated cells (reduced to 20-25%). The values at zero time were obtained by measuring the [^{32}P]-PAR incorporation into proteins before addition of the corresponding electroeluted PARG fractions. The time course of the reaction clearly showed that in untreated cells, 5 minutes were enough for PARG to remove more than 90% [^{32}P]-PAR, whereas the already low levels of [^{32}P]-PAR (20-25%) from treated cells did not change significantly over the whole incubation time with NBD-treated cell purified PARG (Figure 18).

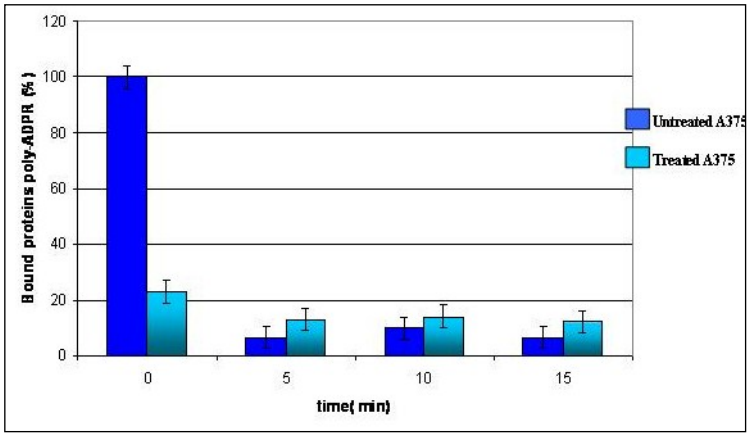

Figure 18: Time course of PARG assay using as substrate PAR-proteins adducts.
100% radioactivity corresponded to 8,000 cpm acid-insoluble material.

Morever, protein-free [^{32}P]poly(ADP-ribose) isolated from untreated and treated A375 cells were analysed by 20% polyacrylamide gel (Figure 19). The results showed different PARP reaction products: a long and complex polymer purified from untreated A375 cells (Figure 19, lane1) and a linear chain of 20-25 ADPR units from treated cells (Figure 19, lane4). Incubation of PARP products from untreated cells with its corresponding purified PARG fractions, gave a complete degradation to ADPR of the polymer (Figure 19, lane2), whereas a scarce hydrolysis occurred when treated-cell polyADPR was incubated with its purified PARG (Figure 19, lane5).

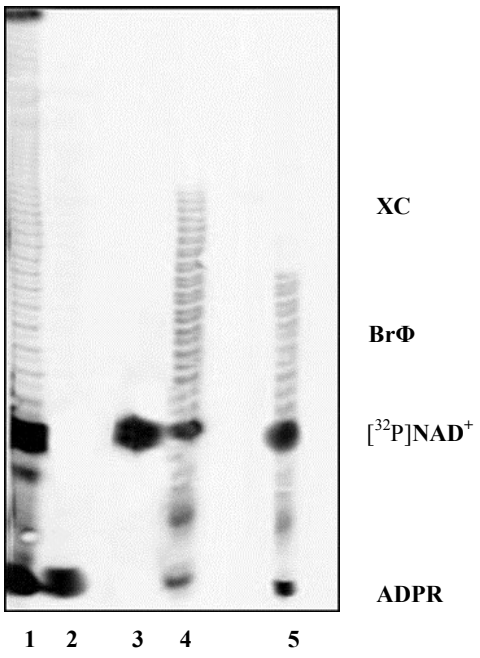

Figure 19: Separation of protein-free [^{32}P]-labelled-polyADPR from untreated and treated A375 cells and their digestion products. Treatment was with 50μM NBD-peptide for 6h. XC, xylene cyanol blue dye; BrΦ, bromophenol blue dye; 1= PAR from A375 cells; 2= PAR from A375 cells after PARG assay; 3= [^{32}P] NAD^+; 4= PAR from A375 treated cells; 5= PAR from A375 treated cells after PARG assay.

Our experiments showed that despite PARG activity is lower in treated A375 cells than in untreated ones, a single PARG isoform was found. This result suggests that the altered PARG activity could depend on the ongoing of the apoptotic process.

3.6 NBD-treatment of different melanoma cell lines results in apoptosis

On the basis of previous results, the effects of NBD- peptide treatment (50µM for 6h) on SK-MEL2, 397, SK-MEL5, M14, LOX, SK-MEL28, UACC257 cells were studied by using biochemical analyses described in the previous sections.

FACS analysis clearly showed that also in these melanoma cells NBD-peptide is able to activate the caspase3 to induce the apoptotic process (Figure 20).

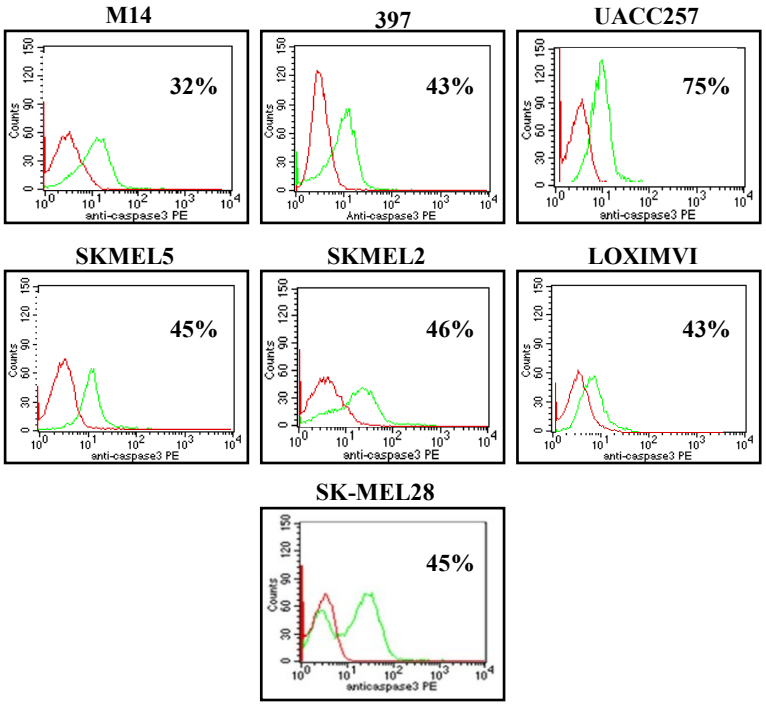

Figure 20: Cytometry analysis. Cell lines were analyzed using the monoclonal antibody (mAb) PE-conjugated anti-human-active-caspase3.

We also tested the production of cleaved fragment of PARP-1(89kDa) due to caspase3 activity. Immunoblotting of samples showed the lack of the 113kDa band corresponding to native PARP-1 and the simultaneously appearance of its apoptotic fragment (89 kDa) produced by caspase-3 activity in M14, 397, UACC257, after treatment with NBD-peptide (Figure 21B).

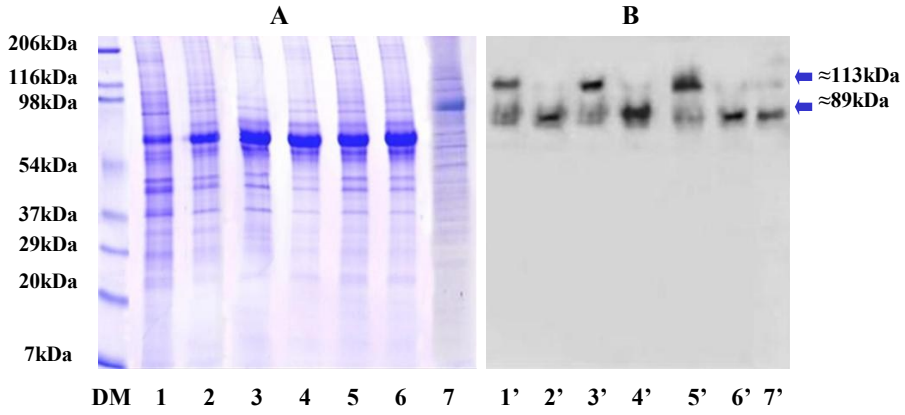

Figure 21: Analysis of nuclear fractions from melanocytes, untreated and treated melanoma cells.
Treatment was with 50μM NBD-peptide for 6 hours. (A) SDS-polyacrylamide gel (12%) stained in Coomassie, (B) Immunoblotting with anti-PARP-1 Abs. 1= M14; 2= treated M14; 3= 397; 4= treated 397; 5= UACC257; 6= treated UACC257; 7= Melanocytes.

Untreated SK-MEL5, SK-MEL2, LOX and SK-MEL28 cell lines showed the 113kDa band of PARP-1 and an additional band of 60kDa, that probably corresponds to PARP-2 protein (Figure 22, lanes 1',3',5',7').

After PARP-1 apoptotic fragment (89 kDa), with no change of the 60 kDa signal (Figure 22B, lanes 2',4',6',8').

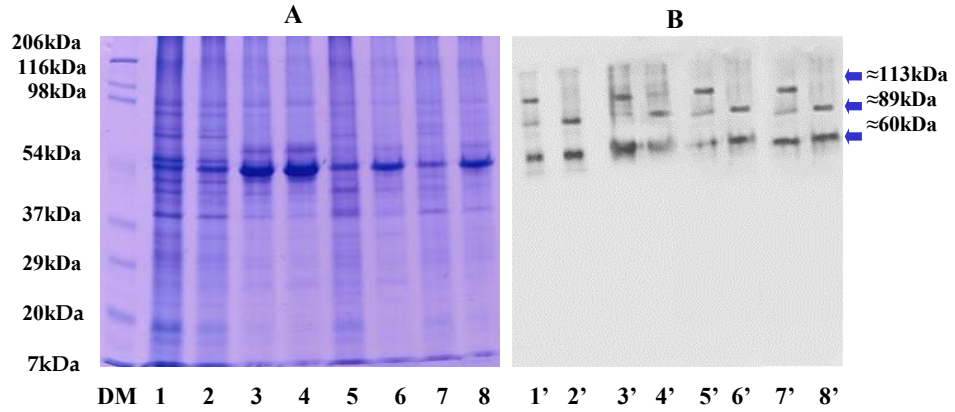

Figure 22: Analysis of nuclear fractions from melanocytes, untreated and treated melanoma cells.
Treatment was with 50µM NBD-peptide for 6 hours. (A) SDS-polyacrylamide gel (12%) stained in Coomassie, (B) Immunoblotting with anti-PARP-1 Abs. Cell lines 1= SK-MEL5; 2= SK-MEL5; 3= SK-MEL2; 4= treated SK-MEL2; 5= LOX, 6= treated LOX; 7= SK-MEL28; 8= treated SK-MEL28.

PARP activity was also measured in all the cells described before and treated with NBD-peptide at 50µM for 6h. The treatment produces an evident decrease of poly(ADPR-ribosyl)ation in all the examined cells (Figure 23).

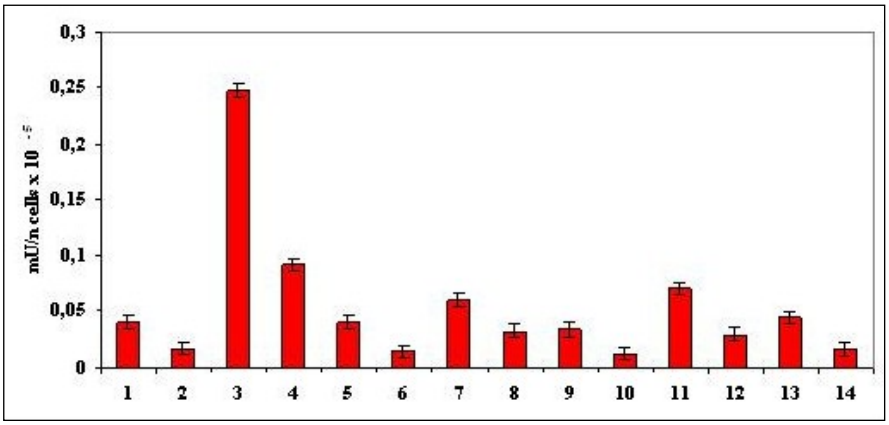

Figura 23: PARP activity before and after treatment with 50µM NBD-peptide for 6h. 1=M14; 2= treated M14; 3= 397; 4= treated 397; 5= UACC257; 6= treated UACC257; 7= SK-MEL5; 8= treated SK-MEL5; 9= SK-MEL2; 10= treated SK-MEL2; 11= LOX; 12= treated LOX; 13= SK-MEL28; 14= treated SK-MEL28.

DISCUSSION

The polyADP-ribosylation reaction results in a unique post-translation modification involved in various cellular processes and conditions, including DNA repair, transcriptional control, genomic stability, cell death and cancer. It has been also reported that this process plays a main role in both anti- and pro-inflammatory events (Hottiger *et al*., 2010., Kiliańska *et al*., 2010). As the inflammation is a critical component in carcinogenesis, recent studies were carried out to define new anti-inflammatory therapies for a complementary approach in treating a variety of tumor types. These observations highlighted NF-kappa B pathway as an attractive way for drug discovery and development (Lin *et al*., 2010). The recent identification of the NEMO-binding domain (NBD) peptide with anti-inflammatory activity, able to block the activation of the IkB kinase (IKK) complex, allowed to hypothesize the apoptosis induction in those tumours, in which NF-kB is constitutively activated (Di Meglio *et al*., 2005).

According to this hypothesis, we treated A375 melanoma cells with NBD peptide. Treatments resulted in a concentration-dependent inhibition of cell proliferation (Figure10). This effect has been associated with the direct inhibition of NF-kB DNA-binding activity (Figure 11), IKK activity (Figure 12), and with caspase3 activation (Figure 13), confirmed by the partial PARP-1 cleavage (Figure 14) and by the decrease of its activity (Figure 15).

Very interestingly, a net immunopositive signal, corresponding to PARP-1 catalytic fragment and a very low polyADP-ribosylating activity have been evidenced in melanocytes too (Figure 14, 15).

These results demonstrate that in melanocyte cells a physiological apoptosis

is active, while, in A375 treated cells, the NBD peptide is able to induce this process.

In the second part of this research, the complete ADP-ribosylating system in NBD-peptide untreated/treated melanoma cells has been described, in order to evidence whether this new potential drug might influence the synthesis and degradation of PAR. Its intracellular levels are important in both death and life of cells (Heeres *et al*., 2007).

In all examined A375 cells and in melanocytes, a single signal was at top of gel, likely PARP-1 itself. (Figure 16).

The different automodification entity (Figure 16), the length and the complexity of polyADPR (Figure 19) seem to influence PAR turnover, guaranteed by a nuclear PARG immunoreactive band of 75kDa (Figure 17).We don't know whether this is a fragment of cytoplasmic PARG (111kDa) translocated to the nucleus (Affar *et al*., 2001) or a nuclear isoform of this enzyme (Tanuma *et al*.,1990). However, our results clearly shown that in untreated cells, the long and branched ADPR polymers (Figure 19) are almost completely digested by PARG enzyme (Figure 19). On the other hand, in treated cells, the short oligomers (about 25-30 units) (Figure 19) are only partially digested (Figure 19).

On the basis of these evidences, we hypothesize that the complete ADP-ribosylating system, including synthesis and degradation of PAR by PARP-1 and PARG respectively, could be involved in the mainteinance of apoptotic process induced by NBD-peptide treatment.

In fact, as high intracellular PAR concentrations are responsible for cell death by necrosis (Ying *et al*., 2001), in treated cells, the low levels of poly-ADP-ribosylated proteins combined with low PARG activity (Figure 18)

might allow the cells to maintain an apoptotic state.

At last, the evidence that in all examined cells, the NBD-peptide induces apoptosis, as it is confirmed by PARP-1, allowed to hypothesize that this drug could represent a new and effective therapy tool for melanoma.

Until now, many drugs able to inhibit NF-kB activity have been tested (Nasr *et al*., 2005., Richardson *et al*., 2004), but one of the most relevant problems of a cancer therapy based on inhibition of NF-kB activity is represented by the difficulty to find compounds which block the oncogenic activity of NF-kB without interfering with its roles in immunity, inflammation and cellular homeostasis. An important aspect of our research is that NBD-peptide does not completely inhibit the transcriptional nuclear factor activity, but produces only its selective modulation. In this way, its physiological functions are not affected.

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