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"Signaling and functions of the calcium/calmodulin-dependent kinase II (CaMKII) in medullary thyroid carcinoma"

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“Signaling and functions of the calcium/calmodulin dependent kinase II (CaMKII) in medullary thyroid carcinoma”

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This dissertation is based upon the following publications:

"Calcium/calmodulin-dependent kinase II (CaMKII) mediates RET-stimulated proliferation in medullary thyroid carcinoma."

Eleonora Russo, Marcella Salzano, Valentina De Falco, Massimo Santoro, Maurizio Bifulco and Mario Vitale *(Manuscript in preparation- This dissertation is the main body of this manuscript)*

"Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates Raf-1 at serine 338 and mediates Ras-stimulated Raf-1 activation."

Salzano M, Rusciano MR, **Russo E**, Bifulco M, Postiglione L, Vitale M.

Cell Cycle. 2012 Jun 1;11(11):2100-6. Epub 2012 Jun 1.PMID:22592532 *(attached at the end)*

Russo E, Salzano M, Postiglione L, Guerra A, Marotta V, Vitale M.

"Interferon-γ inhibits integrin-mediated ERK activation stimulated by fibronectin binding in thyroid cells." J Endocrinol Invest. 2012 Oct 1. [Epub ahead of print] *(attached at the end)*

Russo E, Guerra A, Marotta V, Faggiano A, Colao A, Del Vecchio S, Tonacchera M,Vitale M.

"Radioiodide induces apoptosis in human thyroid tissue in culture."

Endocrine. 2013 Mar 31. [Epub ahead of print] *(attached at the end)*

Salzano M, **Russo E**, Postiglione L, Guerra A, Marotta V, Esposito S, Vitale M. "Interferon-γ inhibits integrin-mediated adhesion to fibronectin and survival signaling in thyroid cells." J Endocrinol. 2012 Dec;215(3):439-44. doi: 10.1530/JOE-12-0335. Epub 2012 Oct 1. *(attached at the end)*

LIST OF ABBREVIATIONS:

CaMKII: calcium-calmodulin dependent kinase II

hCaMKIINα;β: endogenous inhibitor of CaMKII isoform α and β

RET: REarranged during Transfection

MTC: medullary thyroid carcinoma

FMTC: familial medullary thyroid carcinoma

MEN 2A, MEN 2B: multiple endocrine neoplasia type 2A and 2B

PCR: polymarase chain reaction

CT: calcitonin

PTC: papillary thyroid carcinoma

FTC: follicular thyroid carcinoma

ATC: anaplastic thyroid carcinoma

ZD6474: Vandetanib

CycD: cyclin D

KN93: CaMKs pharmacological inhibitor

ABSTRACT

Germline point mutations of the RET gene (REarranged during Transfection) are present in about 70% of sporadic medullary thyroid carcinoma (MTC), a malignant tumor that arises from calcitonin-secreting C cells of thyroid gland, and in almost 100% of the dominantly inherited multiple endocrine neoplasia (MEN) type 2A and 2B and the familial medullary thyroid carcinoma (FMTC). The calcium-calmodulin dependent kinase II (CaMKII) is an ubiquitous serine/threonine protein kinase involved in multiple signalings and biological functions. In epithelial and mesenchimal cells, CaMKII participates with Ras to Raf-1 activation, phosphorylating Raf-1 at S338, a phosphorylation necessary for ERK activation upon different physiologic and pathologic stimuli in the mitogen activated protein kinase (MAPK) cascade. In papillary thyroid carcinoma, CaMKII is activated by $BRaf^{V600E}$, oncogenic Ras and by RET rearrangements (RET/PTC) and participates to the activation of the ERK pathway by oncogenic Ras and RET/PTC, thus modulating tumor cell proliferation. The main aim of this thesis is to determine whether CaMKII is involved also in MTC harboring activating point mutation of RET. Recently it has been shown that an endogenous inhibitor of CaMKII (hCaMKIIN) is expressed in several cell types. Its expression is negatively correlated with the severity of human colon adenocarcinoma, suggesting a pivotal role of CaMKII in the development and progression of carcinomas with oncogenic activation of the MAPK pathway. To determine the role of CaMKII in the RET signaling and in MTC, two activated RET mutants (C634W and M918T) have been expressed in NIH-3T3 cells, observing the following CaMKII activation. MTC cell lines (TT and MZ-CRC1) harboring the most frequent MEN2A and MEN2B mutations (C634W and M918T respectively), have been treated with the CaMKII selective inhibitor KN93 and the following effects on the MAPK pathway and cell cycle have been monitored. Accordingly with results of the RET mutant expression in NIH-3T3, CaMKII was activated in MTC cell lines. Inhibition of CaMKII in these cells induced a decrease of Raf-1 phosphorylation at ser338. Accordingly, also the dephosphorylation of MEK and ERK was observed. CaMKII inhibition was followed by a reduced cyclin D and p27 accumulation, and by a reduction of cell proliferation. These results demonstrate that CaMKII is involved in cell cycle and proliferation in MTC cell lines harboring the RET oncogene. To confirm the actual role of CaMKII in the development and progression of MTC, I determined the relative hCaMKIINα mRNA expression in primary MTC tumors and its correlation with some clinicopathological parameters at surgery. In MTC affected patients hCaMKIINα mRNA expression was inversely correlated with serum calcitonin, tumor extension, tumor staging and presence of metastatic lymph nodes. The results of this thesis indicate that CaMKII has a role in cancers harboring oncogenic point mutation of RET and could represent a new therapeutic target for pharmacological intervention in these tumors.

1.INTRODUCTION

1.1 THYROID CARCINOMAS

The thyroid gland consists of two lobes, connected by an isthmus and consists of two types of cells: the epithelial follicular cells, arranged in spheres around a protein-rich colloid, forming the follicle a functional unit of the gland. Follicular cells take up iodide, which is oxidized to active iodine and incorporated into thyroglobulin. The combination of two iodinated tyrosine residues is required for the synthesis of the thyroid hormones tetra-iodothyronine or thyroxine (T4) and in the liver and kidney, T4 is further converted to T3, the biologically active hormone. Thyroid hormones activate transcription by binding to nuclear hormone receptors, regulating protein synthesis and metabolism in many different organs (Braverman and Roti, 1996).
The second cell type are the neuroendocrine C-cells, embryonically derived from the neural crest. C-cells when stimulated by calcium produce and secrete the polypeptide hormone calcitonin (CT), a 32 amino acids long protein, which inhibits osteoclast secretory activity (Fugazzola et al., 1994).
The majority of thyroid cancers originate from follicular cells; papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) defined well differentiated carcinomas and the undifferentiated anaplastic thyroid carcinoma (ATC)(Santoro et al., 2006).
Papillary thyroid carcinoma is the most frequent form of thyroid carcinoma representing the 80% of all thyroid tumors. The incidence of PTC is correlated to the ionizing radiation exposition. Molecular biology studies of post-Chernobyl thyroid tumors revealed a high prevalence of rearrangements of the *RET* proto-oncogene (57–76%) in PTC (Grieco et al., 1990). The most common genetic alterations in PTC include *BRAF* and *RAS* point mutations, and *RET/PTC* rearrangements which are involved in the Ras/Raf/MAPK signal pathway. The substitution of the valine with a glutamic acid at position 600 of BRAF ($BRAF^{V600E}$ mutation) strongly increases its kinase activity eliciting ERK1/2 phosphorylation (Wan et al., 2004). Chromosomal rearrangements that generate the juxtaposition of the C-terminal region of the RET protein with an N-terminal portion of another protein (RET/PTC rearrangement), can also lead to the constitutive activation of the RET kinase and the downstream signaling including PI3K/Akt, MAPK, JNK, and PLCγ (Knauf et al., 2003).
Follicular thyroid carcinoma (FTC) represents the 10-30% of thyroid tumors. The most frequent genetic alterations include point mutations of Ras and the PAX8/PPARγ rearrangement. Anaplastic thyroid carcinoma (ATC) is the most rare thyroid tumor (2-5% of thyroid tumors) and is undifferentiated. ATC cells are not capable to synthesize thyroglobulin and to pick up iodine. Patients affected by ATC have a poor diagnosis, 75% of which present metastasis at diagnosis. ATC may have Ras, BRAF, β-catenin and p53 mutations. Recent evidence suggest that

one subset of anaplastic thyroid carcinomas are derived from papillary or follicular carcinomas due to BRAF and p53 mutations (Quiros et al., 2005).
A germline point mutation in the *RET* gene on chromosome 10q11.2 is responsible for the hereditary MTC(Nose, 2011). About 5-10% of thyroid cancer is represented by medullary thyroid carcinoma (MTC), which originates from the C-cells. Medullary thyroid carcinoma (MTC) is a C cell-derived calcitonin-producing tumor, that occurs either in sporadic (75% of all MTC) or in familial forms (25%). CT detection in tumor biopsies and elevated plasma CT levels are hallmarks of MTC (Bussolati et al., 1969; Melvin and Tashjian, 1968). All principle oncogenes find in thyroid carcinomas are reported in Table1.

Tumor type	**Oncogene**
PTC	RET/PTC
	NTRK1
	BRAF
	PI3KCA
	RAS
FTC	PAX8/PPARγ
	RAS
	PI3KCA
ATC	RAS
	BRAF
	PI3KCA
	RET/PTC
	CTNNB1
MTC	RET

Table 1. Major genetic alterations in thyroid cancer

The hereditary form of MTC is a dominantly inherited cancer syndrome known as familial medullary thyroid carcinoma (FMTC) when solitary, or multiple endocrine neoplasia type 2A and 2B (MEN2A, MEN2B) when occurs associated to other endocrine tumors derived from neural ectoderm (Gardner et al., 1993).
MTC are known to spread to lymph nodes in the neck and mediastinum at early stages, and eventually also to distant sites, like bone, liver and lung. The classification of MTC is based on the pathological Tumor, Node, Metastases system (pTNM) and is also referred to as stage I (tumor less than 2 cm in diameter without evidence of disease outside of the thyroid gland), stage II (any tumor between 2 and 4 cm without evidence of extrathyroidal disease), stage III (any tumor greater than 4 cm, or level VI nodal metastases or microscopic extrathyroidal invasion regardless of tumor size) and stage IV (any distant metastases, or lymph node involvement outside of level VI, or gross soft tissue extension). The appropriate initial treatment for patients who are diagnosed with

MTC is total thyroidectomy and careful lymph node dissection of the central compartment of the neck (Moley and Fialkowski, 2007). Measurement of postoperative plasma CT levels is a sensitive method to determine whether the operation has been curative (Lips et al., 2001). In contrast to the thyroid follicular cells, C-cells do not take up and store iodine. For this reason, in contrast to papillary and follicular thyroid carcinoma, additional treatment with radioactive iodine is not effective for MTC (Saad et al., 1983). Furthermore, chemotherapy and radiotherapy are usually ineffective for MTC (Marsh et al., 1995). An effective systemic treatment in addition to surgery is currently not available for MTC patients.

1.2- RET *(REarranged during Transfection*)

The *RET* protooncogene has 21 exons distributed over 60 kb and encodes for a receptor tyrosine kinase, which is expressed in neuroendocrine cells (including thyroid C cells and adrenal medullary cells), neural cells (including parasympathetic and sympathetic ganglion cells), urogenital tract cells, and testis germ cells. RET protein is structured with an extracellular portion (which contains four cadherin-like repeats, a calcium binding site, and a cysteine-rich region), a transmembrane portion, and an intracellular portion, which contains two tyrosine kinase subdomains (TK1 and TK2) that are involved in the activation of several intracellular signal transduction pathways.
Ligand stimulation leads to activation of the RET receptor with dimerization and subsequent autophosphorylation of intracellular tyrosine residues, which serve as docking sites for various adaptor proteins (Santoro et al., 2004). Normally, RET is activated by binding of a ligand-coreceptor complex. The family of RET-coreceptor ligands consists of glial cell-line derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). The coreceptors, GDNF family receptor alpha (GFRα)-1, GFRα-2, GFRα-3 and GFRα-4, belong to a glycosyl-phosphatidyl inositol (GPI)-linked receptor family. GDNF primarily associates with GFRα1, whereas neurturin, artemin, and persephin preferentially bind GFRα2, GFRα3, or GFRα4, respectively. RET protein dimerization results in autophosphorylation of several intracellular RET tyrosine residues. Ten autophosphorylation sites are found on both major RET isoforms (RET9 and RET51), and an additional two are found on the longer isoform, RET51. Several are binding sites for a variety of docking proteins. The tyrosine Y1062 has been shown to bind Src homology and collagen (SHC), insulin receptor substrate 1 and 2 (IRS1/2), fibroblast growth factor receptor substrate 2 (FRS2), and protein kinase Cα (PKCα). These proteins are able to activate multiple signaling pathways, including mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, RAS/extracellular signal regulated kinase (ERK), and Rac/c-*jun* NH, kinase (JNK). These pathways are mediators of cell motility, proliferation, differentiation, and survival. DOK 1/4/5/6 (downstream of kinase

1/4/5/6) also binds phosphorylated Y1062, and DOK4 binding has been implicated in GDNF-dependent outgrowth. Binding of c-Src or SH-2Bβ to phosphorylated Y981 promotes survival and differentiation. Other binding sites have also been shown to be important. SHC preferentially binds to activated RET outside lipid rafts, whereas FGF receptor substrate 2 (FRS2) preferentially binds when RET is within the raft. FRS2 activates ERK through both Grb2 and Shp2. The tyrosine Y1015 has been shown to bind phospholipase C γ (PLCγ), then subsequently generates inositol phosphates, modulates the intracellular calcim concentration and activates the protein kinas C (PKC).

In PTC, RET can be activated by chromosomal rearrangement known as RET/PTC rearrangement. In RET/PTC, the 3′ portion of the RET gene is fused to the 5′ portion of various unrelated genes. At least 13 types of RET/PTC have been reported to date, all formed by the RET fusion to different partners (de Groot et al., 2006b).

The two most common rearrangements, *RET/PTC1* and *RET/PTC3*, account for the majority of all rearrangements found in papillary carcinomas. *RET/PTC1* is formed by fusion with the *H4* (D10S170) gene, and *RET/PTC3* by fusion with the *NCOA4 (ELE1)* gene (Pasini et al., 1995). Several studies suggest that the oncogenic effects of RET/PTC require signaling along the MAPK pathway and the presence of the functional BRAF kinase. Indeed, *BRAF* silencing in cultured thyroid cells reverses the RET/PTC-induced effects (Mitsutake et al., 2006).

Papillary carcinomas with *RET/PTC* rearrangements typically present at younger age and have a high rate of lymph node metastases, classic papillary histology, and possibly more favorable prognosis, particularly those harboring *RET/PTC1*. In tumors arising after radiation exposure, *RET/PTC1* was found to be associated with classic papillary histology, whereas *RET/PTC3* type was more common in the solid variants (Nikiforov et al., 1997; Powell et al., 1998). Several studies demonstrated that *RET/PTC* rearrangement could be a unique marker for papillary thyroid carcinoma, however *RET/*PTC has been found in benign lesions including Hashimoto's thyroiditis (HT) and adenomas (Elisei et al., 2001; Ishizaka et al., 1991; Nikiforova et al., 2002). The finding that *RET/PTC* expression is not an absolute PTC marker raises concerns on its clinical utility in inconclusive cytology. Moreover, the finding that *RET* rearrangements can occur only in a fraction of the cells in some PTC raises the alternative hypothesis that *RET/PTC* may also be a common secondary event in the process of thyroid carcinogenesis (Unger et al., 2004).

The subclonal occurrence of *RET* rearrangement in PTC can influence the sensitivity of some methods and might explain why the reported prevalence of *RET/PTC* in papillary carcinomas varies in different studies from 0 to 87%. (Tallini and Asa, 2001; Zhu et al., 2006).

Very recent studies demonstrated that *RET* rearrangement in benign thyroid nodules is not an uncommon occurrence and suggested that its presence could be

associated with a faster nodular enlargement (Guerra et al., 2011; Marotta et al., 2010; Sapio et al., 2011).

Somatic *RET* point mutations have been identified in about 50% of patients with sporadic MTC (Chiefari et al., 1998). MEN2A, MEN2B and FMTC represents the familial forms of MTC. MEN2A is associated with mutations involving the extracellular cysteine codons 609, 611, 618, 620 (exon 10) 630, or 634 (exon 11). The mutations associated with FMTC involve a broad range of codons including some associated with MEN2A, particularly, 609, 618, and 620, as well as others: 768, 790, and 791 (exon 13), 804 and 844 (exon 14), or 891 (exon 15) (Wells and Santoro, 2009). In 95% of patients with MEN2B there is a point mutation in codon 918 (exon 16, Met918Thr) within the intracellular domain of *RET* (Myers et al., 1995). A few patients with MEN2B have a mutation in codon 883 (exon 15) (Gimm et al., 1997). Single MEN2B patients with double RET mutations Val804Met + Ser904Cys and Val804Met + Tyr806Cys have been reported (Iwashita et al., 2000). In MEN2A and FMTC the *RET* mutations lead to a ligand-independent homodimerization and constitutive kinase activity such that *RET* becomes a dominant oncogene. In MEN2B *RET* mutations activate the RET receptor in its monomeric state, leading to phosphorylation of Y1062 and other tyrosines, and also causing a change in substrate specificity (Baloh et al., 1998). Phosphorylation of tyrosine 1062 (Tyr1062) located in the RET carboxyl-terminal tail is important for transforming activity of both RET-MEN2A and RET-MEN2B mutant proteins (Asai et al., 1996). Tyr1062 acts as a docking site for many adaptor or effector proteins such as SHC, FRS2, DOK1/4/5/6, IRS1/2, Enigma, protein kinase C α (PKCα) and Shank (Schuetz et al., 2004). Among these, SHC binding plays a crucial role in activation of both the RAS/ERK and phosphatidylinositol 3-kinase (PI3)-K/AKT pathways (Besset et al., 2000). When SHC binds to phosphorylated Tyr1062, Grb2/SOS and Grb2/GAB complexes are recruited to SHC, leading to activation of the RAS/ERK and PI3-K/AKT pathways, respectively. In addition, Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK) and ERK5 pathways are also activated via Tyr1062 (Murakami et al., 2002) although the precise activation mechanisms remain elusive. (Figure 1)

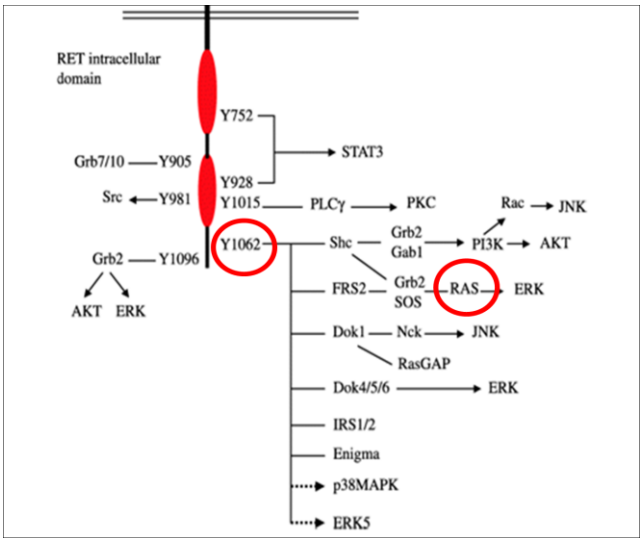

Figure 1. Principle docking sites for intracellular pathways in RET receptor *(de Groot et al., 2006a)*

It has been shown that AKT and JNK phosphorylation was increased in RET-MEN2B-expressing cells compared to RET-MEN2A-expressing cells(Murakami et al., 1999). In addition, it was reported that the JNK pathway is involved in the ability of RET-MEN2B to metastasize (Marshall et al., 1997). These findings suggest that high levels of AKT and JNK activation may be associated with the aggressive properties of MEN2B.

RET gene mutations have a high phenotype-genotype correlation, corresponding with MTC behavior and directly affecting treatment and surveillance. Generally, the least aggressive tumors arise in FMTC, which is characterized by only the development of MTC without other abnormalities. MEN2A tumors are slightly more aggressive, and patients may develop pheochromocytomas, parathyroid hyperplasia, and rarely, cutaneous lichen amyloidosis. MEN2B tumors are the most aggressive, and the syndrome is characterized by pheochromocytomas, skeletal abnormalities, mucosal neuromas, and a marfanoid habitus but not parathyroid hyperplasia. Presently, 500 to 1,000 MEN2 families are recognized worldwide (Brandi et al., 2001). The optimal treatment for MTC in patients with MEN2 is prophylactic thyroidectomy, ideally just prior to extra-thyroidal spread. Because of a good correlation between MTC clinical aggressiveness and the specific *RET* genotype, the timing of surgical intervention varies depending on the specific mutation. The American Thyroid Association has recently refined the categorization of all known mutations into four levels to recommend an age for prophylactic surgery (American Thyroid Association Guidelines Task et al., 2009). Patients with the highest risk are in level D, with mutations in codons corresponding to MEN2B, and should have surgery by age 6 months. Level C

consists of mutations in codon 634, and patients should have prophylactic surgery before age 5 years. Level B consists of mutations in codons 609, 611, 618, 620, and 630. Surgery should be considered before age 5 years, but may be delayed if stringent criteria are met (normal serum calcitonin, normal neck ultrasound, and less aggressive family MTC history). Level A mutations are characterized by MTC with the least aggressive behavior, and surgery may be delayed after age 5 years on the basis of the stringent criteria previously described and the clinician's discretion. (Figure 2).

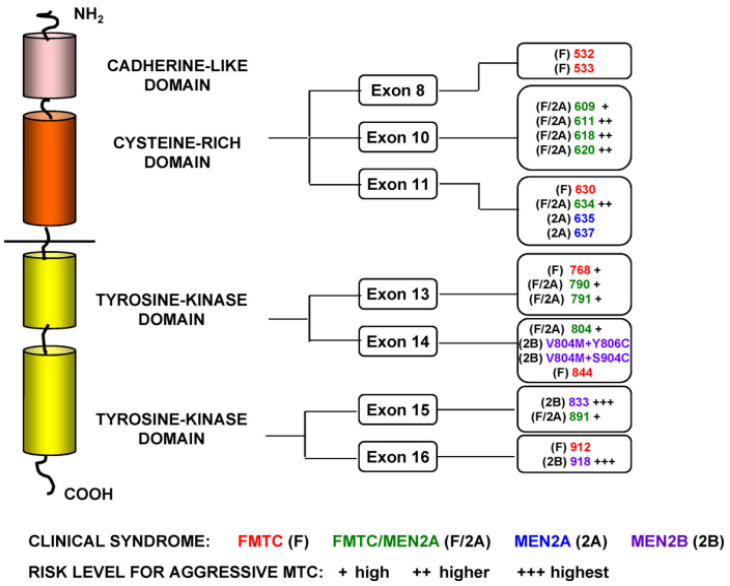

Figure 2. Schematic diagram of the RET receptor and distribution of mutated codons associated with different risk levels for aggressive MTC in MEN2 syndromes. The most common MEN2-associated mutations are reported. Other rare germline or somatic mutations, alone or in combination, have been found at different RET codons. *(Lanzi et al., 2009)*

Several multi-kinase inhibitors have significant activity against RET. Several have shown inhibition of RET kinase and tumor growth in preclinical models of MTC. Vandetanib (ZD6474) was originally developed as a second generation epidermal growth factor receptor (EGFR) TKI, but subsequently was found to have more potent inhibitory effects against VEGF receptor (VEGFR; IC_{50} = 40 nM) and RET (IC_{50} = 130 nM) than EGFR (IC_{50} = 500 nM) (Schlumberger et al., 2008). Vandetanib blocks autophosphorylation of codon 918 mutant RET kinase in intact cells (Carlomagno et al., 2002). Certain mutations in RET codons 804 and 806 have been shown to confer resistance to vandetanib, which may be a concern for

secondary resistance to the drug (Carlomagno et al., 2004). Sorafenib (BAY 43-9006) is another multikinase inhibitor targeting RET, as well as BRAF, VEGFR, and platelet-derived growth factor receptor (PDGFR). *In vitro*, sorafenib inhibits oncogenic RET kinase with an IC_{50} of < 50 nM and decreased tumor volume of TT cells (MTC cell line harboring a codon 634 RET mutation) in athymic mice (Carlomagno et al., 2006).

Overall success seen in these trials represents a major breakthrough in treatment of patients with widespread metastatic MTC. However, current clinical trials of RET-targeted therapies are only the first step in discovering effective therapies for patients with MTC. Further progress in understanding the molecular pathogenesis of MTC is critical to elucidate the role of the RET kinase signaling pathway in tumor progression and maintenance, other critical targets or signaling pathways important in MTC, and mechanisms of primary and secondary resistance to TKIs by potential redundant signaling pathways or by developing "resistance" mutations in RET (Phay and Shah, 2010).

1.3- THE CALCIUM CALMODULIN DEPENDENT KINASES (CAMKs)

Calcium is an ubiquitous intracellular messenger responsible for controlling numerous cellular processes including fertilization, mitosis, neuronal transmission, contraction and relaxation of muscles, gene transcription, and cell death. At rest, the cytoplasmic calcium concentration [Ca]i is approximately 100 nM, but this level rises to 500–1,000 nM upon activation. In osteoblasts, the elevation of [Ca] i is a result of an increase in the release of calcium from endoplasmic reticulum and/or extracellular calcium influx through voltage gated Ca channels. Many of the cellular effects of calcium are mediated by the calcium binding protein, calmodulin (CaM) (Hook and Means, 2001).

The predominant intracellular receptor for Ca^{2+} is calmodulin (CaM), a small, highly conserved Ca^{2+} sensor that is ubiquitously expressed in mammalian cells (Bito, 1998). CaM serves as a receptor to sense changes in Ca^{2+} concentration and, in this way is the second messenger of these ion. Ca^{2+} binds to CaM by a structural motif called EF-hand, and a pair of these structures are located in both globular ends of the protein. When the four binding sites are filled, CaM undergoes a conformational change exposing a flexible eight-turn α helix, which separates the hydrophobic pockets that form in each of the globular ends of the protein. CaM thus become "loaded" with Ca^{2+} and capable to interact with one of its many target protein in the cell. The interaction with target proteins, while usually of high affinity, is rapidly reversible upon a decline in Ca^{2+} concentration (Means, 2000). One of the major family of Ca^{2+}/CaM effectors is represented by the Ca^{2+}/CaM dependent protein kinases (CaMKs) which can be divided into dedicated CaMKs that phosphorylate a single specific substrate such as myosin light chain kinase (MLCK) and the multifunctional CaMKs including CaMKI, CaMKII and CaMKIV that phosphorylate a large number of protein (Braun and Schulman, 1995).

All of the CaMKs, except CaMKIII, have similar overall domain organizations of their 50–60 kD subunits and crystal structures for CaMKI and CaMKII (Hudmon and Schulmann, 2002) have been published. CaMKII contains a unique C-terminal subunit association domain and electron microscopy reveals that it exists as heteromeric dodecamers of α, β, γ, and δ subunits with two hexameric rings stacked one on top of the other (Gaertner et al., 2004). This complex oligomeric structure allows for unique regulatory mechanisms and protein-protein interaction domains that are essential to its functionality, especially in paradigms of learning and memory (Lisman et al, 2002). Activation of CaMKII by Ca^{2+}/CaM allows intramolecular autophosphorylation of several sites, including Thr286, Thr305, and Thr306. Autophosphorylation of Thr286 has two primary consequences: (1) the subsequent dissociation of bound Ca^{2+}/CaM (i.e., when intracellular Ca^{2+} concentration is reduced) is decreased by several orders of magnitude, thereby prolonging its activation, and (2) even after full dissociation of Ca^{2+}/CaM, the kinase retains partial (30%–60%) activity (i.e., Ca^{2+}-independent or

constitutive/autonomous activity) Thus, transient elevations of intracellular $[Ca^{2+}]_i$ can result in prolonged CaMKII activity until protein phosphatases dephosphorylate Thr286 (Colbran,2004). The mechanism of activation of CaMKII is illustrated in figure 3.

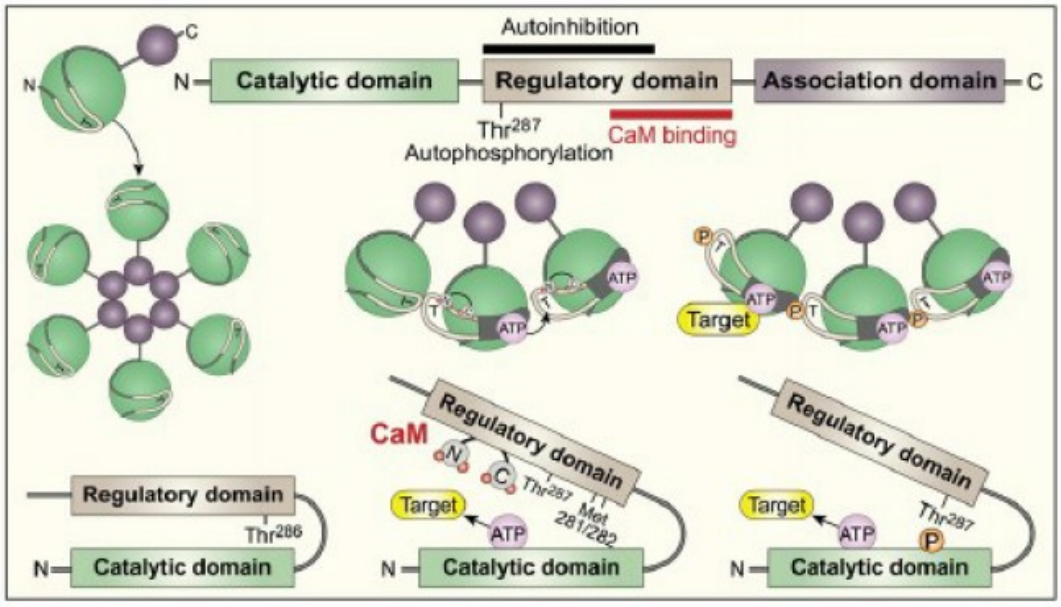

Figure 3. Ca^{2+}/CaM-dependent kinase II structural domains and activation. Under resting conditions the catalytic domain is constrained by the regulatory domain. After intracellular Ca^{2+} rises the Ca^{2+}/CaM binds to the C terminal portion of the CaMKII regulatory domain to prevent autoinhibition of the regulatory domain on the catalytic domain, activating CaMKII. With sustained Ca^{2+}/CaM or increased oxidation, CaMKII transitions into a Ca^{2+}/CaM-autonomous active enzyme after autophosphorylation (at Thr 287) or oxidation (at Met281/282) of amino acids in the regulatory domain *(Anderson, 2005)*

Moreover, the extent of CaMKII autonomous activity can be dictated by the frequency of Ca^{2+} oscillations (Rich and Schulman,1998). This mechanism is thought to be critical in several physiological situations, especially potentiation of synaptic transmission during learning and memory (Lisman et al., 2002). Thus, transgenic mice in which Thr286 is mutated to Ala or Asp exhibit multiple behavioral and learning deficits. Members of the CaMK cascade (CaMKK α and β, CaMKI α, β, δ, and γ, and CaMKIV) are monomeric and, apart from activation by Ca^{2+}/CaM, show very different modes of regulation by phosphorylation compared to CaMKII. These CaMKs, like most other Ser/Thr protein kinases, have an "activation loop" phosphorylation site that is absent in CaMKII. Binding

of Ca^{2+}/CaM to CaMKI and CaMKIV exposes this activation loop site to allow phosphorylation by the upstream CaMKK when simultaneously activated by Ca^{2+}/CaM. Phosphorylation of the activation loop in CaMKI and CaMKIV primarily increases their Ca^{2+}/CaM-dependent activities. CaMKIV, but not CaMKI, can also exhibit significant Ca^{2+}-independent activity (Tokumitsu et al., 2004) In neurons, CaMKK-mediated phosphorylation/activation of CaMKIV appears to be quite transient, lasting for only a few minutes (Uezu et al., 2002) whereas CaMKI phosphorylation can persist up to an hour or more (Schmitt et al., 2005). Although CaMKI and CaMKIV have overlap in substrate specificity determinants, they can also exhibit unique phosphorylation site preferences even in the same protein substrates (Schmitt et al., 2005). Cellular substrate specificities of CaMKI and CaMKIV are often dictated by different subcellular localizations.

The primary substrates of CaMKK are CaMKI and CaMKIV, but CaMKK can also activate PKB/Akt (Yano et al., 1998) and AMP-kinase. PKB and AMP-kinase are referred to as secondary substrates because their phosphorylation/activation by CaMKK is very slow. For example, activation of CaMKI upon NMDA receptor stimulation occurs in less than 5 min, whereas PKB/Akt activation by CaMKK is maximal in about 60 min (Schmitt et al,2005). CaMKK-mediated activation of PKB upon prolonged elevation of $[Ca^{2+}]_I$ results in PKB phosphorylation and inactivation of the proapoptotic factor BAD, thereby protecting neurons from apoptosis(Yano et al., 1998).AMP-kinase is critical for regulation of cellular energy metabolism in many tissues, and its function(s) in brain is just beginning to be explored.

Apart from the activation features, the CaMKs differ from tissue and subcellular localization. CaMKII and CaMKI are ubiquitously expressed, while CaMKIV is tissue specific and expressed mainly in brain, thymus, testis, ovary, bone marrow and adrenal gland (Wang et al., 2001). While CaMKIV is predominantly nuclear (Jensen et al., 1991) and CaMKI appears to be a cytosolic enzyme(Jensen et al., 1991; Picciotto MR, 1996; Picciotto et al., 1995), the subcellular distribution of CaMKII can vary (Heist and Schulman, 1998). Four types of subunits of CaMKII have been identified (α, β, γ, δ) that are encoded by different genes with differing tissue-specific expression. Alternative splicing within the C-terminal sequence of each gene produces further isoforms. Although the biochemical characteristics of CaMKII purified from many tissues are practically identical, the subunit composition, that is dependent on the source, seems to determine the subcellular localization of the complex. The most studied forms of CaMKII in the nervous system are rich in α and β subunits, and are mainly cytoplasmic enzymes. Some splice variants of the α, γ and δ CaMKII genes contain a nuclear localization signal (NLS) (Brocke et al., 1995; Srinivasan et al., 1994), resulting in targeting of the kinase to the nucleus. Expression of the δ isoform together with cytoplasmic isoforms of CaMKII can direct the heteromultimeric enzyme complex to the nucleus, suggesting that the relative abundance of cytoplasmatic or nuclear-targeted subunits may determine the subcellular localization (Heist et al., 1998).

The nuclear entry of CaMKII can also be regulated by other kinases. In all mammalian nuclear CaMKII isoforms, the NLS is followed by a string of four Ser residues. Phosphorylation of the four Ser by CaMKI or CaMKIV blocks both nuclear translocation of α subunit and binding of CaMKII to importin (Griffith et al., 2003).

CaMKII exerts a broad range of biological function such as regulation of gene expression, cell cycle, proliferation and a number of neuronal functions.

CaMKII is one of the most abundant proteins in the brain, comprising 1% of the total protein in the forebrain and 2% in the hippocampus, a region associated with memory. The first of the CaMKII isoforms to be identified, $CaMKII_{\alpha}$ is a major component of the postsynaptic membrane (PSD) in pyramidal neurons. In the PSD, CaMKII is thought to increase synaptic strength by phosphorylating ion channels and signalling proteins such as glutamate receptors and N-metyl D-aspartate (NMDA) receptors (Cruzalegui and Bading, 2000). Thus, CaMKII is involved both in the maintenance of dendritic architecture and synaptic plasticity. In addiction CaMKII is required for long lasting changes in synaptic strength such as long-term potentiation (LTP), a process involved in learning and memory (Giese et al., 1998).

One of the studies that have addressed the nuclear functions of CaMKII was based on the Ca^{2+} stimulation of immediate early genes that are regulated by cAMP response element (CRE) such as c-fos (Means,2000). Phosphorylation of CREB (CRE binding protein) on Ser133 is essential for transcription because it is required for binding of the ubiquitously expressed CREB binding proteins CBP and p300. (De Cesare et al., 1999) Ser-133 was originally identified as the target of protein kinase A (PKA), thus explaining the role of cAMP in transcriptional activation. However CaMKII can also phosphorylate this residue leading to the speculation that CAMKII mediates Ca^{2+} requirement for expression of the immediate early genes (Means,2000). Interestingly, the nature of the effects of CaMKII on transcription seems to be both cell and promoter dependent (Nghiem et al., 1994).

Studies from multiple groups have identified an association between CaMKII and heart disease, suggesting that CaMKII signaling may provide a unique opportunity for the development of novel therapies. The use of inhibitors to block CaMKII activity has been instrumental in understanding this enzyme's role in Ca^{2+} signal transduction. To examine the role of CaMKII specifically in signal transduction, reagents that possess relative specificity for the isoforms of CaMKII were identified. A family of reagents is known as the KN series of inhibitors, with KN-62 being the first described: an isoquinolinesulphonamidederivative ²1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-l-tyrosyl]-4-phenylpiperazine´ with a *K*i value of 0.9 lM. KN-93, a methoxybenzenesulphonamide, has improved solubility with a slightly better inhibitory potency (*K*i= 0.37 lM). Otherwise many intracellular kinases contribute to inhibit CaMII like PKA, the cyclin-dependent kinases, and the mitogen-activated protein kinase JNK.

Moreover, the KN drugs interfere competitively with activation by CaM, and thus they do not inhibit the autonomous activity of the kinase (Sumi et al., 1991; Tokumitsu et al., 1990).
Endogenous inhibitors of CaMKII were first identified in 1998 and 2001 from brain rat extracts, in Cos-7 cell line and in neurons. Up to now, four endogenous CaMKII inhibitory proteins (CaMKIINs) have been identified. Rat CaMKII inhibitory protein α (rCaMKIINα) and β (rCaMKIINβ), both identified from rat brain and human CaMKII inhibitory protein α (hCaMKIINα) and β (hCaMKIINβ) were highly selective in inhibiting CaMKII activity, and some biological functions have been elucidated (Ma et al., 2009; Wang et al., 2008). Identification of the T-site as the CaM-KIIN interaction site on CaMKII provided two mechanisms for this novel differential inhibitor effect: CaMKIIN was competitive with the region around T286 in an isoforms independent manner, and strengthened the CaM binding required for presentation of T286 as a substrate. The natural CaMKII inhibitors protein hCaMKIINs provides a promising alternative to pharmacological inhibitors because they potently inhibits CaMKII but not CaMKI, CaMKIV, PKA, or PKC (Chang et al., 1998, 2001). The two CaMKIIN isoforms (α and β)are highly homologous to each other and colocalize with microtubules in neurons. Both bind selectively to CaMKII only in its activated states (Chang et al,1998; 2001). CaM-KIIN–derived peptides could provide superior CaMKII inhibitors, especially if they are short enough to be synthesized easily.

1.4-CAMKII AND hCaMKIIN IN THE MAPK SIGNALING IN NORMAL AND CANCER CELLS

In the last decade, studies performed in the laboratory of Prof. Vitale, demonstrated that the MAPK pathway activated by several stimuli, is controlled by CaMKII through the regulation of Raf-1 activity. Fibronectin (FN) binding to integrins in thyroid cells, activates the Ras/Raf/MEK/ERK pathway, through the formation of the FAK/Grb-2/Sos complex. Concurrently, integrins binding to FN increases the intracellular Ca^{2+} concentration, that leads to a Ca^{2+}/CaMKII signal. Integrin activation induces Raf-1 and CaMKII to form a protein complex, indicating that intersection between Ras/Raf/MEK/ERK and Ca^{2+}/CaMKII signaling pathways occurs at Raf-1 level. Inhibitory experiments demonstrated that the Ca^{2+}/CaMKII signal is necessary for ERK activation in this context. Interruption of the Ca^{2+}/CaMII pathway using pharmacological (KN93) or peptidic (ant-CaNtide) inhibitors of CaMKII, arrested cell proliferation induced by FN in thyroid cells (Illario et al., 2003; Illario et al., 2005)(figure 4). The cross talk between CaMKII and MAPK pathway, has been demonstrated also in human fibroblasts and in L6 skeletal muscle cells following insulin stimulation. In these cells, insulin induces activation of CaMKII and its association with Raf-1. This event is necessary for the following ERK dependent-DNA synthesis,

demonstrating the role of CaMKII also in the selective control of insulin signaling (Illario et al., 2009). A very recent paper demonstrates that the molecular event following CaMKII/Raf-1 binding is the Raf-1 phosphorylation at S338 (Salzano et al., 2012). This phosphorylation is a necessary step of Ras-mediated Raf-1 activation, occurring upon different physiologic and pathologic stimuli of the MAPK cascade.

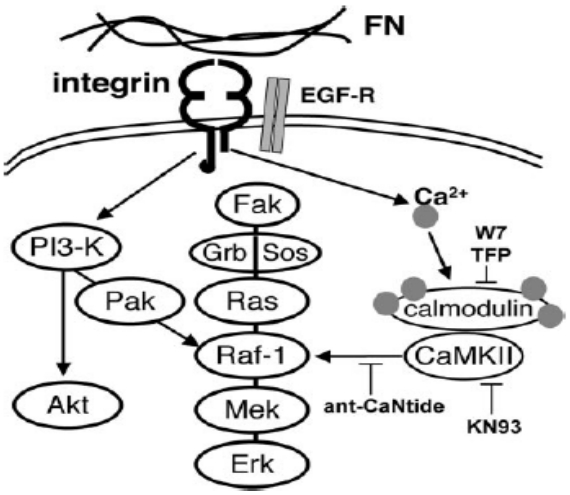

Figure 4. CaMKII binding to Raf-1 is necessary for activatation of the Ras/Raf/MEK/ERK pathway after fibronectin (FN) stimulation in thyroid cells *(Illario et al,2003)*

In most of the cells, in the absence of extracellular stimuli (i.e. hormones, cytokines, integrins), CaMKII is not activated or is in a minimal activation status. This kinase has been found activated in basal unstimulated conditions in some tumors. CaMKII has been found constitutively activated in absence of any stimulation in primary cultures of PTC and in PTC cell lines harboring the oncogenes RET/PTC1 or BRafV600E. The expression of recombinant RET/PTC3, BRafV600E or RasV12 in COS-7 cells, induced CaMKII activation, in a phospholipase C/Ca^{2+} dependent manner. In the PTC cell line TPC-1, harboring RET/PTC1, CaMKII inhibitors attenuated ERK activation and DNA synthesis, indicating that CaMKII is a component of the ERK signal cascade in this cell line (figure 5). Taken together, these data demonstrated a new role of CaMKII in the modulation of tumor cell proliferation and that the PLC/CaMKII pathway could therefore provide appropriate targets for therapeutic intervention of tumors harboring RET/PTC (Rusciano et al., 2010).

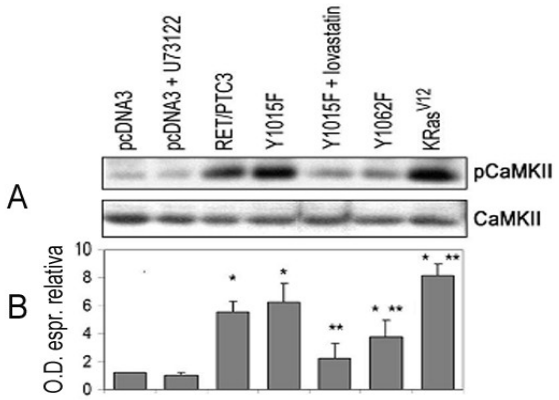

Figure 5. Oncogenic RET/PTC and KRas^{V12} activate CaMKII in Cos-7 cell line *(Rusciano et al,2010)*

A role for CaMKII in the regulation of cell cycle and cell proliferation have been recently extended to other cellular systems, extending the biological role of this kinase.

Tombes et al. demonstrated that CaMKII mediates Ca^{2+}/CaM-dependent G1 phase progression in fibroblasts. Indeed, the inhibition of the endogenous cellular CaMKII by KN93 completely prevented DNA synthesis and led to decreased levels of cyclin D1, a critical regulatory molecule of the G1 phase protein kinase cdk4. More recent studies showed that CaMKII regulates cell proliferation in different cell types. The role of CaMKII has been investigated in prostate carcinoma and has been shown that AR-positive prostate cancer cells can escape apoptosis after inhibition of the CaMKII mediated PI3K-independent activation of Akt (Rokhlin et al., 2007). Moreover, CaMKII can affect apoptotic response of prostate cancer cells by an Akt-independent mechanism. KN93 sharply decreased the level of anti-apoptotic protein Mcl-1 whereas different inhibitors of PI3K/Akt pathway did not change the Mcl-1 expression. In the same cell system, KN93 induced p53 expression and p53-dependent pro-apoptotic protein PUMA (Rokhlin et al., 2010). In colorectal carcinoma Wnt-specific inhibitors SFRP and DKK-1 can block the ability of CaMKII to trigger TCF-1 export (Najdi et al., 2009). In the hepatoma cancer cell line Hep3B, overexpression of constitutively active CaMKII enhanced HIF-1α activity and the CaMKII inhibitor KN93 counteracted this effect. The involvement of CaMKII in HIF-1α activation was also demonstrated in macrophages. CaMKII inhibitors SMP-114 and KN93 down-regulated HIF-1α and VEGF in THP-1 monocytic cells (Westra et al., 2009) However, the role of CaMKII in HIF-1α regulation remains controversial because a specific CaMKII inhibitor SMP-114 had no effect on HIF-1α and VEGF expressions in rheumatoid synovial fibroblasts (Westra et al.,2009). Therefore, the CaMKII regulation of HIF-1α is a cell type-dependent event. All these data demonstrate the pivotal role

of CaMKII in most important signalings ruling the cell fate of normal and tumoral cells.

More recently, an important cancer regulatory role has been proposed for the endogenous inhibitors of CaMKII (hCaMKIINα and β). It has been proposed that hCaMKIINα has a biological role in colo-rectal carcinogenesis (Wang et al., 2008). In this study, hCaMKIINα expression induced a decrease of ERK activity, accumulation of p27 and arrest of cell cycle. In this paper, the authors also showed that hCaMKIIN expression in primary colon adenocarcinoma was negatively correlated with the severity of the disease. Ma et al. demonstrated that intratumoral gene transfer of hCaMKIINβ inhibited the growth of human ovarian cancer *in vivo* and that hCaMKIINβ overexpression affected the expression of cell cycle- and apoptosis-related proteins, and Akt/HDM2 pathway in human ovarian cancer. Also in ovarian adenocarcinoma hCaMKIINβ expression was negatively correlated with the severity of the disease, suggesting that this could be a more general phenomenon. These observations, let hypothesize that hCaMKIINα and β-mediated CaMKII inhibition might provide a promising approach for the drug design of novel cancer therapeutics (Ma et al., 2009; Wang et al., 2008).

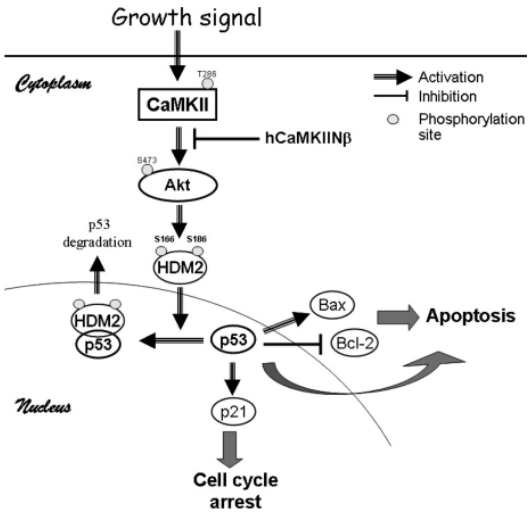

Figure 6. A model depicting the mechanisms for the induction of human ovarian cancer cell cycle arrest and apoptosis by hCaMKIINβ-mediated inhibition of CaMKII.

Inhibition of CaMKII by hCaMKIINβ inactivates PI3K/Akt, which de-regulates the HDM2 expression, leading to the stabilization of p53 protein. p53 then regulates the transcription of target genes, such as p21, resulting in the cell cycle interference. On the other hand, p53-dependent induction of Bax and direct inhibition of Bcl-2, as well as p53-independent signals, promote the cellular apoptosis. *(Wang et al,2008)*

2.AIM OF THE THESIS

The aim of this doctorate thesis is to clarify the role of CaMKII in medullary thyroid carcinoma, in particular I focused my study on the following specific questions:

1- Whether RET mutants activate CaMKII
2- Whether CaMKII is stably activated in MTC cell lines, and if so, whether CaMKII activation is RET mediated
3- Whether CaMKII is involved in the MAPK signaling in MTC cells and whether it modulates cell cycle and proliferation
4- Whether CaMKIINα mRNA relative expression in primary MTC, correlates with clinicopathological features of the disease

The answers to these questions represent the main body of a manuscript in preparation, however during my doctorate program I have been involved in other projects, the published one are attached at the end of this thesis.

3-MATERIALS AND METHODS

3.1-Cell cultures

Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET/C634R and RET/M918T, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. TT cells were from American Type Culture Collection (Manassas, VA, USA). TTs were derived from the primary tumor of an apparently sporadic MTC. TTs harbor a cysteine 634 to tryptophan (C634W) exon 11 RET mutation (Carlomagno et al., 1995) as well as a tandem duplication of the mutated RET allele (Huang et al., 2003) MZ-CRC1 cells were derived from a malignant pleural effusion from a patient with a metastatic MTC (Cooley et al., 1995). MZ-CRC1 cells revealed a heterozygous (ATG to ACG) transition in RET exon 16 resulting in MEN2B-associated substitution of threonine 918 for methionine (M918T).
TT cells were grown in RPMI 1640 supplemented with 16% FBS (Gibco). MZ-CRC1 cells were grown in DMEM supplemented with 10% FBS. All media were supplemented with 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (Gibco). All cell lines were maintained at 37°C 5%CO2.

3.2-MTT assay

TT and MZ-CRC1 cells were plated at a density of 1×10^4 cells/well in 96-well plates in 100 μL medium. After overnight culture, KN93 was added and the cells were cultured up to 9 days. The medium and the inhibitors were renewed every 3 days. Following the designated treatment, the culture medium was removed and 20 μl of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT) (Sigma Chemical Co., St. Louis, MO), 0,5 mg/mL were added to each well. After 4 h at 37 °C of incubation in darkness, the dissolved MTT crystals were quantitated. Optical densities were obtained using a test wavelength of 490 nm (Dynatech MR5000 microelisa spectrophotometer, Chantilly, VA).

3.3-Western blot

For Western blot analysis, cells were lysed in Laemmli buffer [0.125 mol/liter Tris (pH 6.8), 5% 95 glycerol, 2% sodium dodecyl sulfate(SDS),1%βmercaptoethanol, and 0.006% bromophenol blue]. Proteins were resolved by 7–15% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P; Millipore Corp., Bedford, MA). Membranes were blocked by 5% nonfat dry milk,98 1% ovalbumin, 5% FCS, and 7.5% glycine in PBS, washed, and incubated for overnight at 4 C with primary antibodies and then washed again and incubated for 1 h with a horseradish peroxidase conjugated secondary antibody. Finally, protein

bands were detected by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Computer-acquired images were quantified using ImageJ 1.39u, National Institutes of Health, U.S.A. Anti-mitogen-activated protein kinase (MAPK) (1 : 1000) and anti-phospho-MAPK (1 : 1000), which recognizes p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were rabbit polyclonal antibodies from Cell Signaling (Beverly, MA). Anti-RET (1 : 1000) is a rabbit polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (Santoro et al., 1995). Anti-phospho905 is a phospho-specific polyclonal antibody that recognizes RET proteins that are phosphorylated at Y905 (Carlomagno et al., 2003). Blots were incubated with primary antibodies for 1 hour at room temperature, followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The blots were then incubated with the goat anti-rabbit secondary antibody (1 : 3000) coupled to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Mouse monoclonal antibodies to total- and phospho- ERK-1/2 and polyclonal; antiphospho-CaMKII antibody (pT286-CaMKII) were from Cell signaling; anti-phospho Raf-1 S338 was purchased by Upstate.
Each experiment was performed at least three times.

3.4-Reagents and inhibitors

To inhibit CaMKII activity a pharmacologiacal inhibitor has been used: KN93.
KN93 is a potent, selective and cell permeant pharmacological inhibitor of the CaMKs (IC_{50}=370 nM; Rezazadeh,2006). This drug is an isoquinolonesulfonamides and functions as a competitive ATP antagonist (Tokumitsu et al., 1990).
The CaMK inhibitor KN93 and the CaM inhibitors N-(6 aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7) were purchased from Sigma Aldrich.
ZD6474 (Vandetanib) has demonstrated potent inhibition of ligand-dependent RET receptor tyrosine kinase activity (IC_{50}=100 nM) and selective inhibition of RET-dependent thyroid tumour cell growth *in vitro* (Carlomagno et al., 2004; Carlomagno et al., 2002) ZD6474 inhibited the majority of mutated, activated forms of RET receptor tyrosine kinase and also inhibited the wild-type enzyme. Therefore, in addition to inhibition of VEGFR-2 tyrosine kinase and EGFR tyrosine kinase, inhibition of RET tyrosine kinase by ZD6474 may provide particular additional antitumour effects in the treatment of tumours with genetic changes in the *RET* gene (mutation or translocation) that lead to RET receptor signalling-dependent tumour cell growth (Santoro et al., 2002). ZD 6474 was kindly provided by AstraZeneca (Macclesfield, UK).

Ionomycin (IC50=100nM) is a selective calcium ionophore produced by the bacterium *Streptomyces conglobatus*. It is used as a research tool to understand Ca^{2+} transport across biological membranes.
BAPTA-AM is a highly selective Ca^{2+} chelator over Mg^{2+}. This product has a total of six possible protonation sites. All four carboxyl groups are considered unprotonated at physiologic pH. Ionomycin and BAPTA were provided by Sigma Aldrich.

3.5-RNA extraction and relative mRNA extraction

Total RNA was extracted from TT and MZ-CRC1 cell lines using 500 µl TRI Reagent, resuspended in 10 µl DEPC water, and reverse-transcribed with SuperScript III (Invitrogen, Milan, Italy) in a 20-µL reaction volume with random primers.. mRNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR with the cDNA of the MTC patients. Real-time PCR was performed using SYBR Green PCR reagents (Fermentas) and primers specific for CaMKIIα, hCaMKIINα and β-actin. The CAMKII alpha forward primer (5'to 3') was GGGGGAAACAAGAAGAGC and the reverse primer (5'to 3') was GTGCTCTCTGAGGATTC.
The hCaMKIINα forward primer(5'to 3) was TACGGCGACGAGAAGCTGAG, the reverse (5' to 3') TCAGCACGTCATCAATCCTATC.
The β-actin forward primer (5'to 3') was 5'-TTC CTT CCT GGG CAT GGA GT-3'; the reverse primer (5'to 3') was 5'-TAC AGG TCT TTG CGG ATG TC-3'.
The samples were analyzed with Biorad iCycler with the following protocol: 95°C x 5'; 95°C x10"-60°C x60" (x 40 cycles). The levels of relative mRNA expression were determined by normalizing to β-actin expression and adopting the ΔΔCt method. (Livak et al,2001)

3.6 TNM and staging classification of tumors.

MTC were classified according to the American Joint Committee on Cancer (AJCC) TNM system. T1: The tumor is 2 cm (slightly less than an inch) across or smaller and has not grown out of the thyroid. T2: The tumor is more than 2 cm but not larger than 4 cm (slightly less than 2 inches) across and has not grown out of the thyroid. T3: The tumor is larger than 4 cm across, or it has just begun to grow into nearby tissues outside the thyroid. T4: The tumor is any size and has grown extensively beyond the thyroid gland into nearby tissues of the neck, such as the larynx (voice box), trachea (windpipe), esophagus (tube connecting the throat to the stomach), or the nerve to the larynx. Stage I (T1, N0, M0): The tumor is 2 cm or less across and has not grown outside the thyroid (T1). It has not spread to nearby lymph nodes (N0) or distant sites (M0). Stage II: One of the following applies: T2, N0, M0: The tumor is more than 2 cm but is not larger than 4 cm across and has not grown outside the thyroid (T2). It has not spread to nearby lymph nodes (N0) or distant sites (M0). T3, N0, M0: The tumor is larger than 4 cm

or has grown slightly outside the thyroid (T3), but it has not spread to nearby lymph nodes (N0) or distant sites (M0). Stage III (T1 to T3, N1a, M0): The tumor is any size and might have grown slightly outside the thyroid (T1 to T3). It has spread to lymph nodes around the thyroid in the neck (N1a) but not to other lymph nodes or to distant sites (M0). Stage IV: One of the following applies: T4, any N, M0: The tumor is any size and has grown beyond the thyroid gland and into nearby tissues of the neck (T4a). It might or might not have spread to nearby lymph nodes (any N). It has not spread to distant sites (M0). T1 to T3, N1b, M0: The tumor is any size and might have grown slightly outside the thyroid gland (T1 to T3). It has spread to certain lymph nodes in the neck (cervical nodes) or to lymph nodes in the upper chest (superior mediastinal nodes) or behind the throat (retropharyngeal nodes) (N1b), but it has not spread to distant sites (M0). Any T, any N, M1): The tumor is any size and might or might not have grown outside the thyroid (any T). It might or might not have spread to nearby lymph nodes (any N). It has spread to distant sites (M1).

3.6-Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed by using the t test, ANOVA test and Ranking test for hCaMKIINα experiments. Each experiment was performed at least three times. The level of significance was set at P less than 0.05.

4.RESULTS

4.1-RET MUTANTS ACTIVATE CaMKII BY A Ca^{2+}/CaM-MEDIATED SIGNAL

Murine NIH3T3 fibroblasts were serum starved for 24 hours and then incubated with the calcium ionophore ionomycin 2 μM for 5 minutes. The cells were then lysed in RIPA buffer, the protein were resolved on SDS PAGE, and the phosphorylation of Thr 286 of CaMKII was evaluated by Western Blot with a phospho-specific antibody (p-CaMKII-T286).
In parental NIH3T3 harbouring the wild type RET gene, a minimal CaMKII phosphorylated was observed at basal condition, as compared to the kinase phosphorylation induced by ionomycin treatment (Figure 7A).
NIH3T3 cells stably transfected with the RET mutants RET/C634R, and RET/M918T were starved from serum for 24 hours and then treated with the calcium ionophore ionomycin 2 μM for 5 min and with the calcium chelator BAPTA-AM for 30 minutes at two different concentrations (5 and 10 μM). The cells were then lysed in RIPA buffer and the phosphorylation on thr 286 of CaMKII was evaluated as before (Figure 7B). In the absence of stimuli the activation of CaMKII was evident. The treatment with ionomycin had no effect, while the treatment with BAPTA-AM decreased CaMKII phosphorylation in a dose dependent manner. These results demonstrate that CaMKII is activated by RET mutants in a calcium-dependent manner.

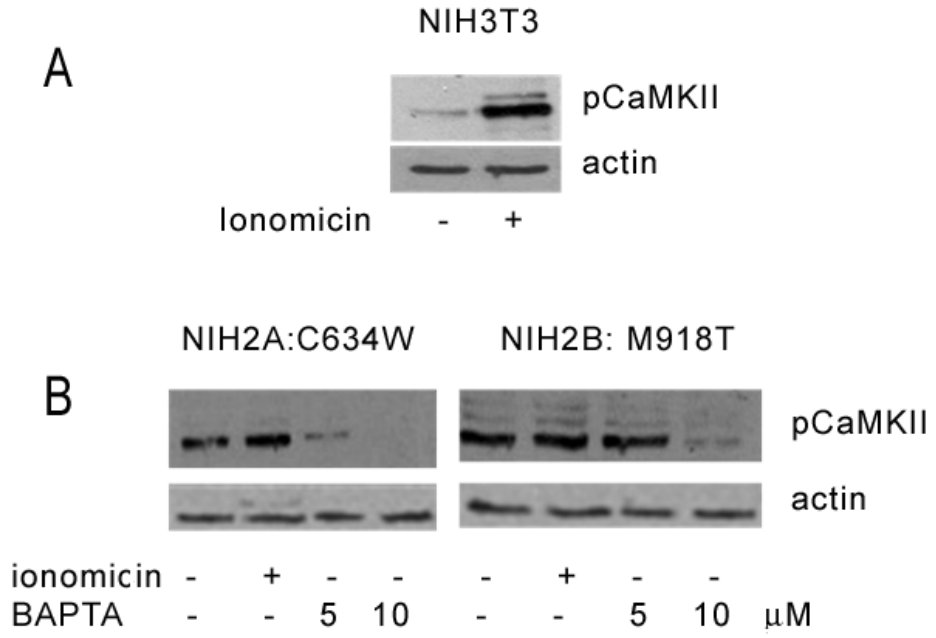

Figure 7- RET mutants activate CaMKII.
Parental murine NIH3T3 fibroblasts (A) and NIH3T3 cells stably transfected with the RET mutants RET/C634R (NIH2A) and RET/M918T (NIH2B) (B) were serum starved for 24 hours and then treated with the calcium ionophore ionomycin 2 μM for 5 minutes or with with BAPTA-AM at different concentration (5, 10 μM) for 30 minutes. The phosphorylation on thr 286 of CaMKII was evaluated by Western Blot with a phosphor-specific antibody (p-CaMKII-T286).

4.2-CaMKII IS ACTIVATED IN MTC CELL LINES

The MTC derived cell lines TT and MZ-CRC1 contain a cysteine 634 to tryptophan (C634W) transversion at exon 11 of the RET gene (Carlomagno et al., 1995) with concurrent tandem duplication of the mutated RET allele (Huang et al., 2003), or a methionine 918 to threonine (M918T) transversion at exon 11 of the RET gene, respectively.
Both cell lines were starved from serum for 24 hours and incubated in suspension with the calcium chelator BAPTA-AM or the ionophore ionomycin for 30 minutes at different concentrations. The cells were then lysed in RIPA buffer, the protein

were resolved on SDS PAGE, and the phosphorylation on Thr 286 of CaMKII was evaluated by Western Blot. (Figure 8)
In unstimulated cells, CaMKII was strongly phosphorylated. Ionomycin treatment produced a paradoxical effect reducing CaMKII phosphorylation. The kinase activation was calcium sensitive as demonstrated by a dose-dependent inhibition with the calcium chelator BAPTA.

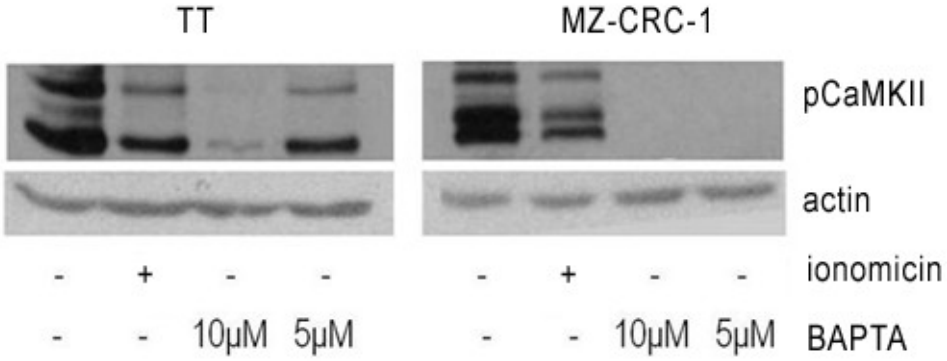

Figure 8. CaMKII is activated in unstimulated MTC cell lines
TT and MZ-CRC1 were starved from serum for 24 hours and incubated in suspension with the calcium chelator BAPTA-AM or the ionophore ionomycin for 30 minutes at the indicated concentrations. Phosphorylation of CaMKII at Thr 286 was evaluated by Western Blot.
In unstimulated cells CaMKII was strongly phosphorilated. Ionomycin treatment produced a paradoxical effect reducing CaMKII phosphorylation. The kinase activation was calcium sensitive as demonstrated by a dose-depemdemt inhibition with the calcium chelator BAPTA.

4.3-CaMKII ACTIVATION IS RET AND PLC-γ MEDIATED IN MTC CELL LINES

TT cells were starved from serum for 24 hours and incubated in suspension with increasing concentration of the RET inhibitor ZD6474 (Vandetanib) for 60 minutes. The cells were then lysed in RIPA buffer, the protein were resolved on SDS PAGE, and the phosphorylation of RET-Y1062, CaMKII and MEK, were evaluated by Western Blot with the phosphor-specific antibodies. (Figure 9 A). Similar results were obtained in MZ-CRC1 cells not shown.
PLCγ is activated by RET and increases intracellular Ca^{2+} concentration. Thus, CaMKII activation by RET might be PLCγ mediated. To elucidate this issue, TT and MZ-CRC1 cells were starved from serum for 24 hours and incubated with

increasing concentrations of the PLC-γ inhibitor U73-122 for 2 hours. The cells were then lysed and the proteins were analyzed by Western Blot (Figure 9B). In MZ-CRC1 cells, 15 μM U73-122 was sufficient to induce a dramatic CaMKII dephosphorylation, whereas in TT cells the same effect was obtained with 45 μM. The different result between the two cell lines evidences a major sensitivity of MZ-CRC1 cell line to calcium deprivation and confirm that in these cells CaMKII activation is RET/ PLCγ mediated.

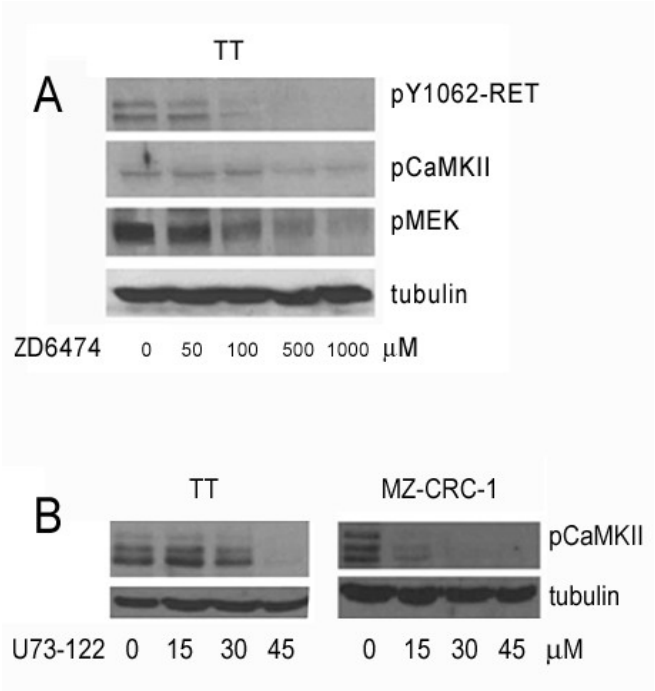

Figure 9. CaMKII activation in TT cells is RET mediated
A, TT cell line was starved from serum for 24 hours and incubated in suspension with increasing concentration of the RET inhibitor ZD6474. The protein were resolved on SDS PAGE, and the phosphorylation of RETY1062, CaMKII and MEK, were evaluated by Western Blot with the phosphor-specific antibodies.
B, TT and MZ-CRC1 were starved from serum for 24 hours and incubated for 2 hours with increasing concentration of the PLC-γ inhibitor U73-122 (15-30-45 μM). The cells were then lysed and CaMKII phosphorylation was evaluated by Western Blot.

4.4-ERK ACTIVATION IN MTC CELL LINES IS CALCIUM-CALMODULIN MEDIATED

TT and MZ-CRC1 cells were serum starved for 24 hours and treated with the calcium chelator BAPTA-AM or the inhibitor of calmodulin W7 at different time (15 and 30 minutes) and concentration (15-30-45 μM). The cells were then lysed in RIPA buffer, the protein were resolved on SDS PAGE, and ERK phosphorylation was evaluated by Western Blot with the phosphor-specific antibodies (Figure 10).
ERK phosphorylation was evident in unstipulated cells and remained unchanged following ionomycin treatment. BAPTA-AM treatment completely abrogated ERK phosphorylation in MZ-CRC1 cells. The ERK phosphorylation inhibitory effect of BAPTA in TT cells was minor and unexpectedly inconstant. In Figure 9 a minor inhibition is displayed. W7 displayed a powerful reproducible inhibitory effect of ERK phosphorylation, demonstrating that CaMKII activation in these cells is calcium/calmodulin dependent.

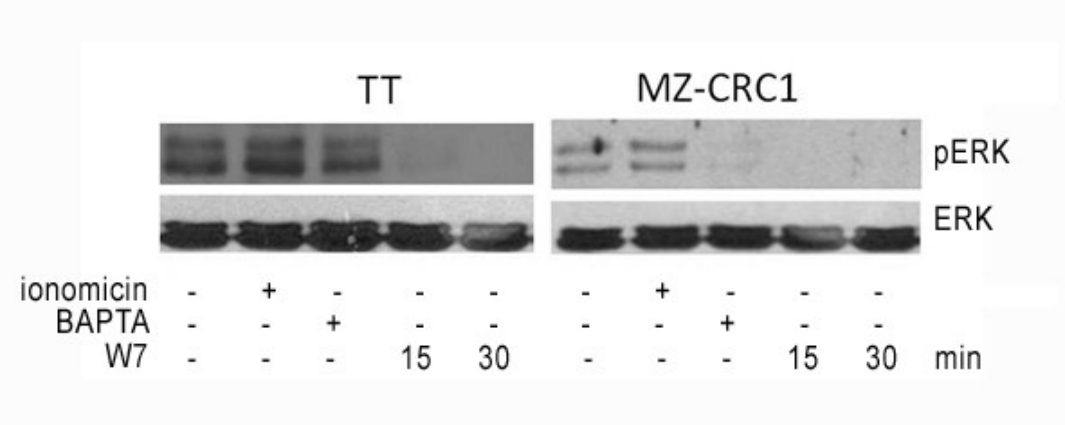

Figure 10. ERK activation in MTC cell lines is calcium/calmodulin mediated
TT and MZ-CRC1 cells were serum starved for 24 hours and treated with the ionomycin or BAPTA-AM for 30 minutes, or with the calmodulin inhibitor W7 for 15 or 30 minutes. The cells were then lysed in RIPA buffer and ERK phosphorylation was evaluated by Western Blot.

4.5-ERK ACTIVATION IN MTC CELL LINES IS CAMKII MEDIATED

TT and MZ-CRC1 were starved from serum for 48 hours and incubated in adhesion with the pharmacological CaMKII inhibitor KN93 10μM for 24 and 48 hours. The cells were then lysed in RIPA buffer, the proteins were resolved on SDS PAGE, and the phosphorylation of RET-Y1062, CaMKII-Thr286, Raf-1 Ser 338 and ERK were evaluated by Western Blot with the phospho-specific antibodies (Figure 11).
KN93 was ineffective on RET phosphorylation, ensuring that the effects on the phosphorylation status of other kinases was not a direct effect on RET. A time-dependent inhibition of CaMKII phosphorylation was evident. A substantial dephosphorylation of Raf-1 at Ser 338 and ERK occurred by 24 h. These results demonstrate that ERK activation in TT and MZ-CRC1 cell is CaMKII mediated.

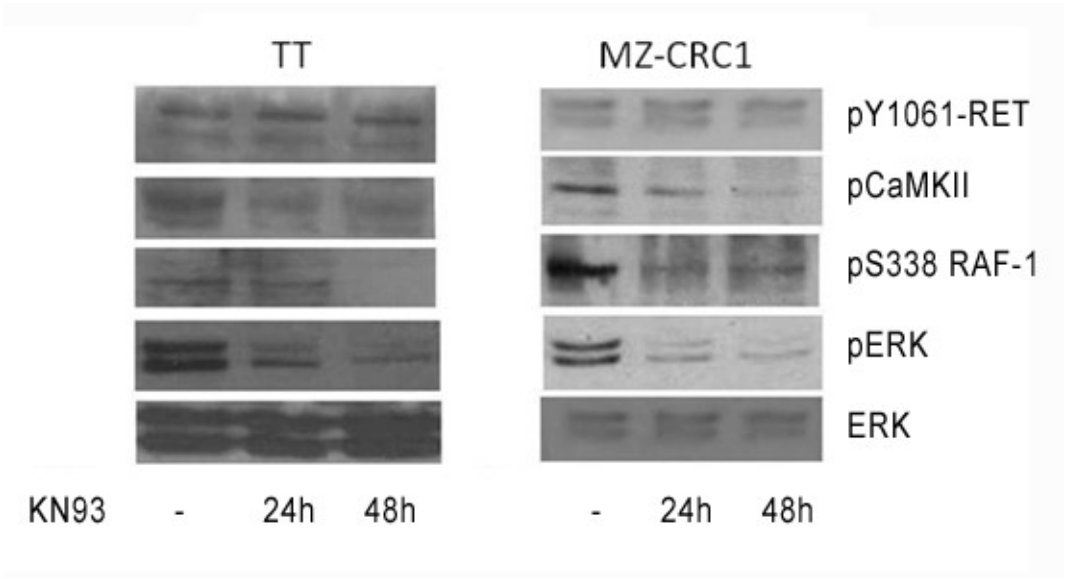

Figure 11. ERK activation in MTC cells lines is CaMKII mediated
MTC cell lines were starved from serum for 48 hours and incubated in adhesion with the pharmacological CaMKII inhibitor KN93 10μM for 24 and 48 hours. The cells were then lysed and the phosphorylation of RET-Y1062, CaMKII-T286, Raf-1-S338 and ERK was evaluated by Western Blot.

4.6- INHIBITION OF CaMKII BLOCKS CELL CYCLE IN MTC CELLS.

TT and MZ-CRC1 were starved from serum for 48 hours and incubated in adhesion with the pharmacological CaMKII inhibitor KN93 for 24 and 48 hours. The cells were then lysed and the levels of cyclin D and $p27^{Kip1}$ were evaluated by Western Blot (Figure 12)
A remarkable decrease of cyclin D expression was evident in both cell lines, with a time dependent fashion. Following the treatment with KN93, a modest accumulation of $p27^{Kip1,}$ more evident in TT cells, could be observed.

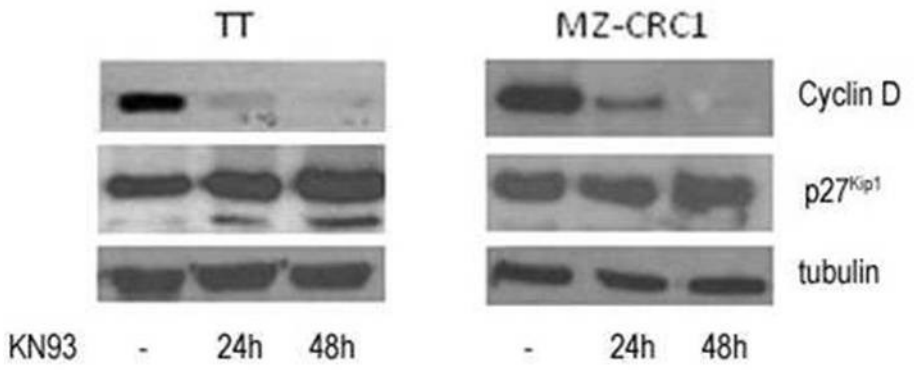

Figure 12. Inhibition of CaMKII modulates cell cycle machinery in MTC cell lines.
MTC cells were starved from serum for 48 hours and incubated with the CaMKII inhibitor KN93 10 μM for 24 or 48 hours. The levels of cyclin D and $p27^{Kip1}$ were evaluated by Western Blot with the specific antibodies.

4.7-CaMKII INHIBITION INDUCES MTC CELL GROWTH ARREST

The MTC cell lines TT and MZ-CRC1 were plated in 96 well 10.000 cells/well and treated with KN93 at different concentration (2.5, 5 and 10 μM) for 9 days. Every three days the medium and the inhibitor were renewed and MTT assay was performed (Figure 13).
In TT cells, a significant reduced number of viable cells was observed after 9 days of treatment with 5 μM KN93. At 10 μM concentration, KN93 strongly inhibited cell viability/proliferation already by 3 days in both cell lines. Microscopic observation of MZ-CRC1 cells treated with 10 μM KN93, revealed a considerable number of floating cells and adherent round shaped cells already by 3 days of

culture, indicating a toxic effect of the treatment. MTT assay indicated a great reduction of viable MZ-CRC1 cells after 9 days of treatment with 10 μM KN93. TT cells appeared more resistant to the toxic effect of KN93.

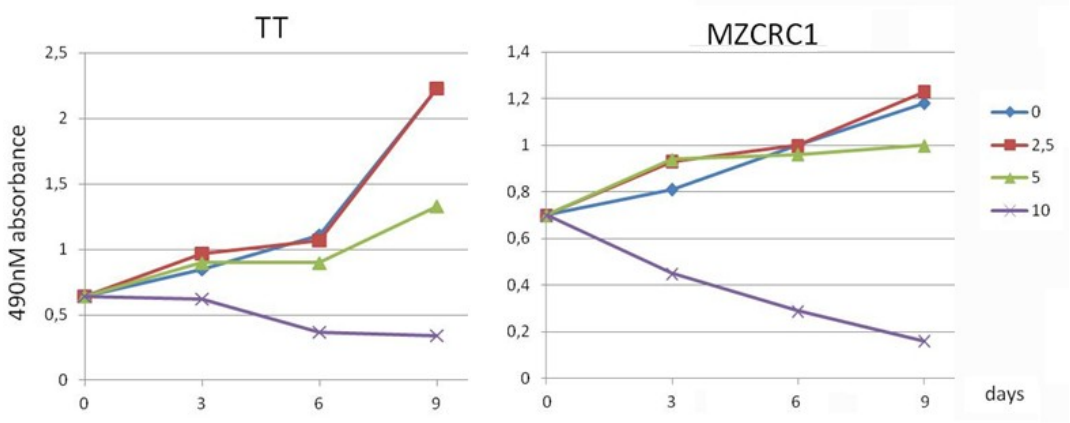

Figure 13. Effects on cell proliferation and viability of CaMKII inhibition.
TT and MZ-CRC1 cells were plated in 96 well 10.000 cells/well and treated with the CaMKII inhibitor KN93 at different concentration (2.5, 5, 10 μM) for 9 days. Every three days the medium and the inhibitor were renewed. At the end of the culture, the plates were incubated with MTT in the dark for 3 hours at 37°C 5% CO_2. The absorbance at 490 nM was recorded and the data were analyzed to report the cell viability.

4.8- hCaMKIINα EXPRESSION IN PRIMARY MTC AND MTC CELL LINES

The hCaMKIINα mRNA relative expression was assessed by real time PCR in 21 tissue samples of primary MTCs. Relative mRNA expression level was determined as previously reported (Livak and Schmittgen, 2001) and normalized with β-actin. hCaMKIINα was found in all tissues with a remarkable variable expression level (Figure 14). hCaMKIINα mRNA expression in MZ-CRC1 was about two fold than in TT cells. This result pursued us to investigate about a possible correlation between hCaMKIINα mRNA expression and clinicopathological features in MTC patients.

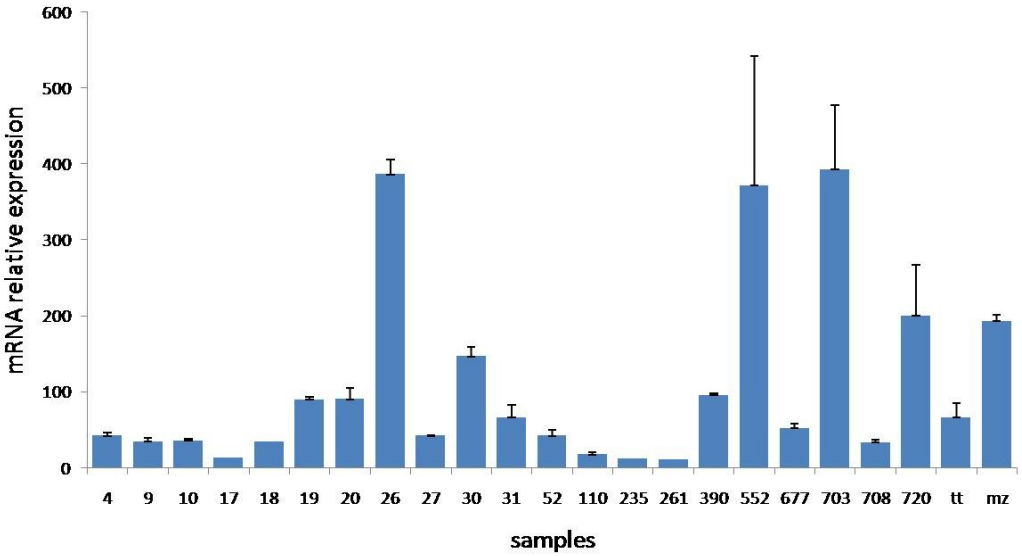

Figure 14. hCaMKIINα expression in primary MTC and MTC cell lines.
hCaMKIINα mRNA relative expression was assessed by real time PCR in 21 tissue samples of MTC. Relative mRNA expression level was normalized with β-actin. The sample with minor relative expression (sample n° 261) was the standard. In MZ-CRC1 was two-fold then TT cells.

4.9- hCaMKIINα EXPRESSION IS INVERSELY CORRELATED WITH SERUM CALCITONIN LEVEL, LOCAL TUMOR EXTENSION, STAGING, AND LYMPH NODE METASTASIS.

The association between clinicopathological characteristics at surgery time and hCaMKIINα mRNA expression was determined. hCaMKIINα mRNA expression was inversely correlate with the serum calcitonin concentration measured at surgery time (R2=0.032 in Spearman rank correlation p=0.017) (Figure 15A). The local tumor extension was classified following the American Joint Committee on Cancer (AJCC) TNM system. hCaMKIINα mRNA expression was significantly correlated to the inverse of the local tumor extension (T) (F=5.276, P=0.0094 by ANOVA) (Figure 14B) and tumor staging (F=5.158, P=0.0043 by ANOVA) (Figure 14C). Inverse correlation was also observed between hCaMKIINα mRNA expression and lymph node metastasis at surgery time. (Student's t-test; p=0.0297) (Figure 14C).
These results indicate that hCaMKIINα expression is inversely correlated with a more aggressive disease at diagnosis, suggesting that inhibition of CaMKII by its endogenous inhibitor protects patients from more aggressive medullary thyroid carcinoma.

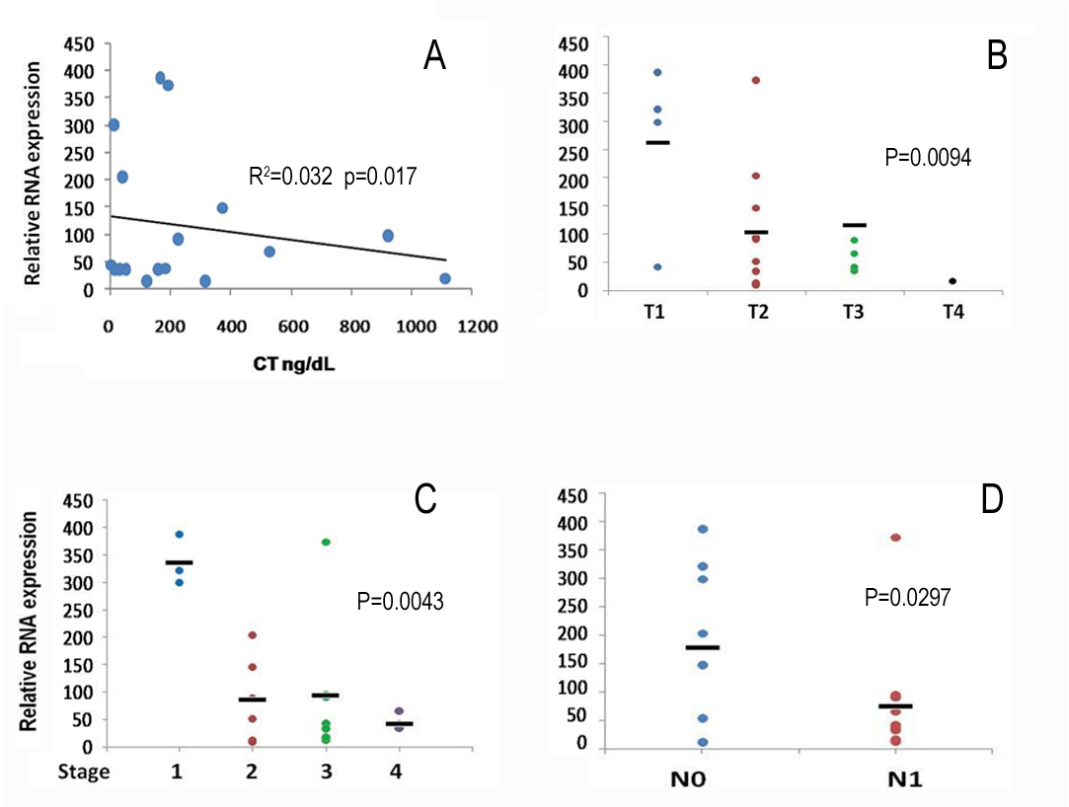

Figure 14. hCaMKIINα expression is inversely correlated with disease extension.

hCaMKIINα mRNA relative expression was assessed by real time PCR in 21 tissue samples of MTC and correlated with serum calcitonin (A); T classification of the TNM system (B); tumor staging (C); lymph node metastasis (D). The ordinates report relative hCaMKIINα mRNA expression. CT, calcitonin; Bars are averages.

5. DISCUSSION AND CONCLUSIONS

MTC is a rare C-cell derived, calcitonin-producing tumor that occurs either in sporadic (75% of all MTCs) as well as in familial forms (25%). Germline point mutations in the *RET* gene on chromosome 10q11.2 are responsible for the hereditary MTC and occurs in about 70% of the sporadic form (Nose, 2011). RET point mutations are associated with the familial medullary thyroid carcinoma (FMTC) and with multiple endocrine neoplasia type 2A and type 2B (MEN2A and MEN2B). MEN2A is associated with mutations involving the extracellular cysteine codons 609, 611, 618, 620 (exon 10) 630, or 634 (exon 11). The mutations associated with FMTC involve a broad range of codons including some associated with MEN2A, 609, 618, and 620, as well as 768, 790, and 791 (exon 13), 804 and 844 (exon 14), or 891 (exon 15) (Wells and Santoro,2009). A point mutation at codon 918 (exon 16, Met918Thr) within the intracellular domain of *RET*, accounts for the 95% of genetic alterations in patients with MEN2B (Myers et al., 1995). A few patients with MEN2B have a mutation at codon 883 (exon 15) (Gimm et al., 1997). In MEN2A and FMTC, *RET* mutations lead to a ligand-independent homodimerization and constitutive kinase activity so that *RET* becomes a dominant oncogene.

All these genetic alterations have in common the ability to confer to RET receptor an constant ligand-independent activity, transforming an hormone receptor into an oncogene. In MEN2B, *RET* mutations activate the RET receptor in its monomeric state, leading to phosphorylation of Y1062 and other tyrosines, (Baloh et al., 1998). Phosphorylation of tyrosine 1062 (Tyr1062) located in the RET carboxyl-terminal tail is important for transforming activity of RET mutant proteins (Asai et al., 1996). Tyr1062 acts as a docking site for many adaptor or effector proteins such as SHC, FRS2, DOK1/4/5/6, IRS1/2, Enigma and protein kinase C α (PKCα) (Schuetz et al., 2004). Among these, SHC binding plays a crucial role in activation of both the RAS/ERK and phosphatidylinositol 3-kinase (PI3)-K/AKT pathways (Besset et al., 2000). When SHC binds to phosphorylated Tyr1062, Grb2/SOS and Grb2/GAB complexes are recruited to SHC, leading to activation of the RAS/ERK and PI3-K/AKT pathways respectively. In summary, ligand-activated RET and oncogenic RET signaling comprises the RAS/RAF/MEK/ERK1/2, the PI3K/Akt, the PI3K/Rac/JNKp38/ERK5, the PLCγ/inositol/Ca^{2+} and PLCγ/PKC pathways.

CaMKII is an ubiquitous serine-threonine kinase, one of the most abundant proteins in the brain, comprising 1% of the total protein in the forebrain and 2% in the hippocampus, a region associated with memory. The first of the CaMKII isoforms to be identified, $CaMKII_{\alpha}$ is a major component of the postsynaptic membrane in pyramidal neurons. CaMKII exerts a broad range of biological function such as regulation of gene expression, cell cycle, proliferation and a number of neuronal functions. A novel function assigned to CaMKII is its

regulatory effect in the MAPK signaling. CaMKII binds to Raf-1 and phosphorylates this kinase at Ser338 (Salzano et al., 2012). This phosphorylation is necessary for Raf-1 full activation and is necessary for up-stream effectors to activate the MAPK kinase pathway leading to ERK1/2 activation. The CaMKII/Raf-1 interplay has important cellular effect on different cellular functions in a cell type-dependent fashion. In thyroid cells stimulated with fibronectin, CaMKII is necessary to integrin-stimulated proliferation and survival (Illario et al., 2003). In L6 skeletal muscle cells and in fibroblasts, CaMKII is necessary to insulin stimulated proliferation and modulates Glut-4 translocation to the cell plasma membrane (Illario et al., 2009).

In different tumors, CaMKII has been found constitutively activated in absence of any stimulation. In PTC primary cultures and in PTC cell lines harboring the oncogenes RET/PTC1 or BRafV600E, CaMKII was activated also in the absence of extracellular stimulation (Rusciano et al., 2010). The expression of recombinant RET/PTC3, BRafV600E or RasV12 in COS-7 cells, induced CaMKII activation, in a PLC/Ca^{2+} dependent manner. Based upon these data, we hypothesized that also in MTC cells, CaMKII might be activated by RET oncogene and might participate to the oncogenic signaling leading to transformation of the C cell. Previous experiments demonstrated that the RET/PTC3 activate CaMKII trough two pathways: Y1062/RAS/PLCγ/Ca2+/CaMKII and Y1015/PLCγ/Ca2+/CaMKII. This conclusion arises from the observation that inhibition of CaMKII activation is achieved by both RAS and PLCγ inhibition and by calcium chelators. The NIH3T3 mutants carrying the RET mutants used in my experiments, provided us with convincing evidence that oncogenic RET activates CaMKII though a calcium mediated signal. In these cells, CaMKII resulted in an active state of a magnitude comparable with that obtained by the calcium ionophore ionomycin, and was inhibited by the calcium chelator BAPTA. However, while the NIH3T3 is a good cell model, suitable to study signal transduction pathways, it is a model too distant from MTC cells and epithelial cells-derived tumors. The biological effects of CaMKII are cell type dependent and results obtained in NIH3T3 cannot be transferred to other cell types without a direct experimental validation. Indeed, inhibition of CaMKII in NIH3T3 and in some prostate cancer cells, does not prevent cell proliferation as observed in other cell types such as normal thyroid cells, thyroid cancer cells of fibroblasts. For this reason I choose to investigate the role of RET-activated CaMKII in MTC cell lines. TT and MZ-CRC1 are the best characterized and most used MTC cells, although these cells are difficult to use because they have a long doubling time and are resistant to most of transfection methods. Another difficulty of my study is represented by the limitation of tools available for CaMKII inhibition. Indeed, because of the expression of four different isoforms and several splicing variants, interfering RNA has a limited efficacy, so that CaMKII inhibition in most of the studies in the literature is obtained pharmacologically by KN93. In most of the

experiments that I performed, the results obtained in the two MTC cell lines were identical or similar, with only a quantitative difference. In both cell lines, CaMKII was in an active state also in the absence of extracellular stimuli. Calcium chelation and calmodulin inhibition abrogated the kinase phosphorylation, indicating that CaMKII in these cells was not mutated and that its activity was depended by a calcium/calmodulin mediated signaling. CaMKII activation was induced by RET mutants C634W and M018T as demonstrated by its inhibition induced by the RET inhibitor ZD6474. The effect of this inhibitor was more evident in TT than in MZ-CRC1 cells, suggesting some difference between the two RET mutants or the existence in MZ-CRC1 cells of an alternative pathway modulating the intracellular calcium concentration. Regardless the mechanisms by which the calcium/calmodulin signaling is generated in these MTC cell lines, the experiments performed with U73-122 demonstrated that CaMKII activation was mediated by PLCγ. As in the other cell types investigated previously, also in MTC cells, active CaMKII binds and phosphorylates Raf-1 at Ser 338, participating to the MAPK pathway, leading to cell proliferation and survival. CaMKII *per se* is not an activator of the MAPK pathway. Indeed, it has been demonstrated that phosphorylation of Ser338 potentiates Raf-1 activation and that full Raf-1 activation is achieved by concurrent S338 and Y341 (i.e. by Src) phosphorylation. Accordingly, direct evidence in COS-7 cells demonstrated that expression of constitutively activated CaMKII did not induce ERK activation. These data, together with the evidence provided by the present study, indicate that CaMKII is required for ERK activation by other signal/s generated by RET mutants in MTC.

A role for CaMKII in the regulation of cell cycle and cell proliferation in some tumors has emerged by studying its endogenous inhibitors. The endogenous inhibitors of CaMKII (CaMKIIN α and β) were first identified from brain rat extracts, in Cos-7 cell line and in neurons. It has been hypothesized that CaMKIIN competes with the region around T286 in an isoforms independent manner (Ma et al.,2009). The endogenous CaMKII inhibitor hCaMKIINα induces accumulation of $p27^{Kip1}$, deactivation of ERK and cell cycle arrest in colorectal carcinoma. hCaMKIINα expression is resulted negatively correlated with the severity of human colon adenocarcinoma, while hCaMKIINβ expression was negatively correlated with the severity of ovarian adenocarcinoma (Ma S et al.,2009).
These evidence suggested that the counteracting role of hCaMKIIN could be a more general phenomenon and that amplifying hCaMKIINα and β-mediated CaMKII inhibition might provide a novel approach for cancer therapy (Ma et al.,2009; Wang et al.,2008)
The analysis of hCaMKIINα expression in 21 MTC revealed a broad variability. Notably, I found a strong correlation between hCaMKIINα expression levels and the disease extension in patients affected by MTC. Although the sample number was low, a highly significant inverse correlation was found between hCaMKIINα mRNA expression in the tumors and the serum calcitonin levels, tumor extension,

staging and lymph node metastasis. All these findings suggest that attenuation of CaMKII activity is correlated with a less aggressive tumor.

These results, when confirmed in a larger cohort of patients, suggest that hCaMKIINα might be used as a prognostic factor useful for tailoring the therapy of MTC. As a final consideration, CaMKII could represent a new therapeutic target for pharmacological intervention in MTC.

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REPORT

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Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates Raf-1 at serine 338 and mediates Ras-stimulated Raf-1 activation

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The calcium/calmodulin-dependent kinase II (CaMKII) participates with Ras to Raf-1 activation, and it is necessary for activation of the extracellular signal-regulated kinase (ERK) by different factors in epithelial and mesenchimal cells. Raf-1 activation is a complex multistep process, and its maximal activation is achieved by phosphorylation at Y341 by Src and at S338 by other kinase/s. Although early data proposed the involvement of p21-activated kinase 3 (Pak3), the kinase phosphorylating S338 remains to be definitively identified. In this study, we verified the hypothesis that CaMKII phosphorylates Raf-1 at Ser338. To do so, we determined the role of CaMKII in Raf-1 and ERK activation by oncogenic Ras and other factors. Serum, fibronectin, Src^{Y527} and Ras^{V12} activated CaMKII and ERK, at different extents. The inhibition of CaMKII attenuated Raf-1 and ERK activation by all these factors. CaMKII was also necessary for the phosphorylation of Raf-1 at S338 by serum, fibronectin and Ras. Conversely, inhibition of Pak3 activation by blocking phosphatidylinositol-3-kinase was ineffective. The direct phosphorylation of S338 Raf-1 by CaMKII was demonstrated in vitro by interaction of purified kinases. These results demonstrate that Ras activates CaMKII, which, in turn, phosphorylates Raf-1 at S338 and participates in ERK activation upon different stimuli.

Introduction

The signaling cascade including Ras, Raf-1, mitogen-activated protein kinase (MEK) and extracellular-regulated kinase (ERK) is involved in the regulation of pivotal cellular processes by different factors.[1,2] Although the Raf proteins have been extensively studied since their discovery over 20 years ago, more is still to be understood about their mechanism of regulation. Raf-1 activation is a complex process involving multiple converging signalings, protein-protein interactions and phosphorylation at multiple sites.[3,4] Phosphorylation is an important mechanism by which Raf-1 activity is regulated. In the canonical model of ERK activation pathway, receptor protein-tyrosine kinases such as the epidermal growth factor (EGF) receptor or the platelet-derived growth factor (PDGF) receptor autophoshorylate, bind and activate the small GTP-binding protein Ras. Ras, in turn, recruits Raf-1 to the plasma membrane, where this kinase is phosphorylated at multiple sites, including S338 and Y341.[5] Phosphorylation at S338 is critical for Raf-1 activation and ERK stimulation by a variety of factors including activated integrins and growth factors.[6,7] Maximal Raf-1 activation is achieved by concurrent phosphorylation at Y341 and S338.[8] While concordant data indicates Src as the kinase phosphorylating Raf-1 at Y341, it is still controversial which is the kinase phosphorylating Raf-1 at S338. In early studies, p21-activated kinase 3 (Pak3) was found to stimulate Raf-1 kinase activity and its phosphorylation at S338, while the same effects induced by Ras^{V12} were inhibited by expression of a dominant-negative Pak3.[9] Phosphatidyl-inositol-3-kinase (PI3-K) inhibitors (Wortmannin and Ly-294002) were shown to inhibit both Raf-1 kinase activity and its phosphorylation at S338, stimulated by EGF and by integrins in COS-7 cells.[6,10] However, a careful re-examination of those results raised some doubts on the effective role proposed for Pak3. Chiloeches et al. argued against the physiological role proposed for Pak3, mainly because supporting data were obtained by overexpression of recombinant factors or by using PI3-K inhibitors at concentrations that block also Ras activity.[11] These authors concluded that Pak3 was not the mediator of S338 phosphorylation in growth factor-stimulated cells, and that this mediator was still to be identified.

We previously demonstrated that the calcium/calmodulin-dependent kinase II (CaMKII) binds Raf-1 upon integrin activation and that it is necessary to ERK stimulation upon different factors in different cell types.[12-16] CaMKII is an ubiquitous serine/threonine kinase whose activation is modulated by intracellular

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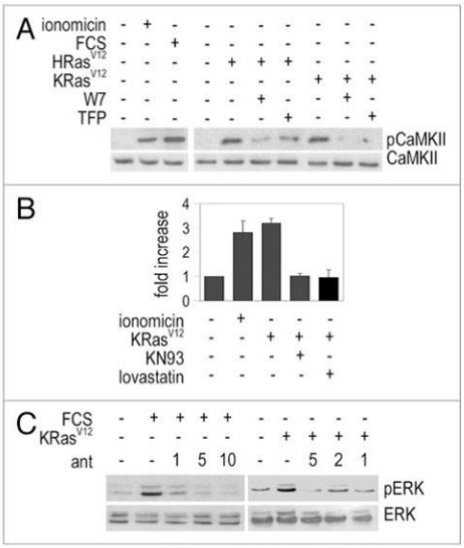

Figure 1. CaMKII is activated by different factors and is necessary for ERK phosphorylation in NIH-3T3 cells. (A) NIH-3T3 cells were starved from serum for 24 h and treated with ionomycin, FCS or transiently transfected with a plasmid encoding oncogenic Ras isoforms HRas^{V12} and KRas^{V12}. Where indicated, the cells were treated for 30 min with 30 μM W7 or 50 nM TFP. CaMKII phosphorylated at T286 (pCaMKII) and total CaMKII were visualized by western blot. (B) NIH-3T3 cells and NIH-3T3 stably expressing KRas^{V12} were starved and treated for 30 min with 2 μM ionomycin, 10 μM KN93 or 5 μM lovastatin. CaMKII activity were evaluated by in vitro kinase activity assay. The results are presented as fold increase of incorporated cpm. Data are reported as the mean ± standard deviation from duplicate experimental points. (C) NIH-3T3 were transfected with a Ras^{V12} expressing plasmid or an empty vector. After serum starvation, the cells were treated with antCaNtide for 30 min at indicated concentration, stimulated with FCS for 30 min (left) and ERK phosphorylation was visualized by western blot.

calcium concentration, hence it is activated by a large variety of factors. It is noteworthy that the CaMKII consensus sequence (R/KXXS/T) in Raf-1 aminoacidic sequence contains S338. In this study, we demonstrate that Ras activates CaMKII, which, in turn, phosphorylates Raf-1 at S338. CaMKII mediates the Ras-stimulated Raf-1 activity and is a key factor in the ERK activation pathway by different stimuli, such as fibronectin-stimulated integrins, serum and Ras and in different cell types.

Results

CaMKII is activated by Ras and is necessary for ERK phosphorylation. We previously demonstrated in epithelial cells (thyroid cells) and mesenchymal cells (myotubes and fibroblasts) that CaMKII is necessary for ERK activation by different factors (integrin activation, insulin, RET/PTC).[14-16] We asked whether CaMKII is activated by Ras and whether it is involved in Ras-induced ERK activation in different cell types. To evaluate whether Ras stimulates CaMKII phosphorylation at Thr286, NIH-3T3 cells were transiently transfected with expression vectors encoding HRas^{V12} and KRas^{V12} isoforms. 48 h post-transfection, serum-starved cells were treated for 30 min with the calmodulin inhibitors W7 or TFP, and CaMKII phosphorylation at Thr286 was visualized by western blot using a phospho-specific antibody (**Fig. 1A**). Ionomycin, a potent calcium ionofore and fetal calf serum (FCS) treatment were used as controls for CaMKII maximal activation. CaMKII phosphorylation was absent in unstimulated cells. Both the oncogenic Ras isoforms induced CaMKII phosphorylation at Thr286. This effect was abrogated by the treatment with W7 and TFP. This result demonstrated that CaMKII is phosphorylated by Ras through a Ca^{2+}/calmodulin-dependent signal. To determine whether Ras induces a continuous activation of CaMKII, we measured CaMKII activity by in vitro assay in NIH-3T3 cells stably transfected with a KRas^{V12} expression vector (**Fig. 1B**). CaMKII activity stimulated by Ras^{V12} was comparable to that induced by ionomycin treatment. The specificity of stimulation was demonstrated by the inhibitory effect of both KN93 and lovastatin (used here as inhibitor of Ras farnesylation). In order to investigate the role of CaMKII in ERK activation by Ras, we tested the effects of CaMKII-specific inhibitory peptide antCaNtide on ERK phosphorylation in NIH-3T3 transiently transfected with KRas^{V12} (**Fig. 1C**). siRNAs are not a convenient tool to abrogate CaMKII activity due to the presence of multiple CaMKII isoforms. Indeed, siRNA only reduced the global cellular CaMKII activity (not shown), while antCaNtide and the CaMKII dominant-negative mutant (K42M, used in subsequent experiments) are recognized as the most specific and potent inhibitors.[17] AntCaNtide pretreatment reduced FCS-induced ERK phosphorylation in a dose-dependent manner. In parallel, ERK phosphorylation induced by KRas^{V12} was inhibited by antCaNtide treatment. These data demonstrate that Ras activates CaMKII, and that, in turn, this kinase is necessary to ERK activation by Ras.

Raf-1 activation by Ras^{V12} is CaMKII-mediated. Because most of the studies on Raf-1 have been performed in COS-7 cells, we choose this cell line to perform experiments useful to provide more insight into the mechanism of Raf-1/CaMKII interplay. In other cell types, endogenous Raf-1, but not B-Raf, formed a complex with activated CaMKII that was precipitated by anti-CaMKII antibodies and recognized by anti-Raf-1 antibodies.[12,13] The selective CaMKII/Raf isoforms interaction was confirmed in COS-7 (**Fig. 2**). Then, we evaluated the ability of Ras and Src to activate CaMKII in these cells. COS-7 cells were transiently transfected with expression vectors for Ras^{V12} and Src^{Y527}, and CaMKII activity was determined by in vitro activity assay (**Fig. 3A**). Both Ras and Src activated CaMKII with a similar intensity, reaching the level of activation induced by ionomycin. Ras and Src cooperate for serine and tyrosine phosphoryations of Raf-1, and both are necessary for its full activation.[8] We determined whether Ras and Src stimulate Raf-1 activation in a CaMKII-dependent manner. COS-7 cells were transiently transfected with expression vectors for Ras^{V12}, Src^{Y527} and CaMKII dominant-negative mutant (CaMKIIdn), and activity of endogenous Raf-1 was measured by kinase assay (**Fig. 3B**). A modest Raf-1 activation

was induced by Src^{Y527} alone, while Ras^{V12} alone was more efficient. Maximal Raf-1 activation was achieved by cooperation of both factors. CaMKII inhibition by CaMKIIdn reduced of about 50% Raf-1 activation induced by Ras^{V12} alone and Ras^{V12} plus Src^{Y527}. $CaMKII^{1-290}$, a truncated form of CaMKII that makes the enzyme constitutively active, potentiated the stimulatory effect of Ras^{V12} plus Src^{Y527} but was unable to activate Raf-1 by itself (**Fig. 3C**). These data demonstrated that Ras^{V12} and Src^{Y527} cooperate to maximal Raf-1 activation, through CaMKII.

CaMKII mediates FCS- and FN-induced phosphorylation of Raf-1 at Ser 338. Seven CaMKII consensus sequence (R/KXXS/T) are present along Raf-1 aminoacidic sequence, and one of these contains S338. Based on previous experiments on the role of Pak, we determined whether phosphorylation of Raf-1 at S338 was CaMKII mediated. To this aim, serum-starved TAD-2 cells were stimulated by FCS or FN for 30 min upon pretreatment with CaMKII inhibitors (KN93 or antCaNtide) or with the PI3-K inhibitor Ly-294002. Raf-1 phosphorylation at S338 was visualized by immunoprecipitation of the kinase and western blot with specific phosphorylated S338-Raf-1 antibody (**Fig. 4**). Both FCS and FN stimulated S338 phosphorylation compared with basal condition. The inhibition of CaMKII reduced S338 phosphorylation by FCS and FN, in a dose-dependent manner. Conversely, PI3-K inhibition was ineffective on S338 phosphorylation. These data demonstrated that Raf-1 phosphorylation at S338 by FCS or FN is CaMKII-dependent in TAD-2 cells.

Phosphorylation of Raf-1 at S338 by Ras^{V12} is CaMKII mediated. To determine whether CaMKII mediates Ras^{V12}-dependent Raf-1 phosphorylation at S338, COS-7 cells were transiently transfected with expression vectors for Ras^{V12}, Src^{Y527} with or without CaMKIIdn. Raf-1 phosphorylation at S338 was visualized by western blot after Raf-1 immunoprecipitation in serum-starved cells (**Fig. 5**). EGF stimulation for 10 min (used as a positive control for maximal Raf-1 phosphorylation), Ras^{V12} and Src^{Y527} induced S338 phosphorylation. Co-expression of Ras^{V12} and Src^{Y527} produced a maximal S338 phosphorylation according to previous studies in reference 8.

Co-transfection of CaMKIIdn together with Ras^{V12} and Src^{Y527} resulted in a 60% reduction of pS338 phosphorylation. Also, the expression of the constitutively activated CaMKII mutant, induced a striking S338 phosphorylation compared with control points. These data demonstrate that CaMKII mediates Ras-induced Raf-1 phosphorylation at S338 and that CaMKII by itself is able to phosphorylate Raf-1 at the same aminoacidic residue.

CaMKII directly binds and phosphorylates Raf-1 at S338. To determine whether CaMKII directly binds and phosphorylates Raf-1 at S338, Raf-1 was immunoprecipitated from unstimulated TAD-2 cells and incubated in vitro for 30 min with active recombinant CaMKII. Phosphorylated Raf-1 at S338 was visualized by western blot with specific antibody. In the presence of a calcium chelator (EGTA), CaMKII is inactive, while it is maximally activated in the presence of calcium. The experiment clearly demonstrated that S338 was phosphorylated in the presence of calcium and in the absence of antCaNtide (**Fig. 6**). As expected, KN93 treatment was ineffective on S338 phosphorylation, because this drug interferes competitively with CaMKII/CaM binding, and thus, it is ineffective on the autonomous activity of the kinase.[17]

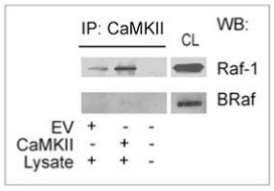

Figure 2. CaMKII/Raf co-immunoprecipitation. COS-7 cells were transiently transfected with empty vector (EV) or a CaMKII expressing vector. An excess of enzyme is sufficient to activate the CaMKII complex also in the absence of a stimulus.[35] After 48 h starvation, cell lysate (CL) or immunoprecipitated CaMKII were submitted to SDS-PAGE, blotted onto nitrocellulose membrane and Raf-1 or B-Raf were detected by specific antibodies.

Discussion

CaMKII is an ubiquitous serine/threonine kinase with a broad range of biological cellular functions, including regulation of gene expression, cell cycle, apoptosis and proliferation.[18,19] This kinase is one of the most abundant protein in the brain, where it is associated to neuronal functions, such as memory. Most of the studies on CaMKII focused on its role in neuronal cells. Our recent studies demonstrated the pivotal role of CaMKII in the Ras-ERK pathway, in different cell models. Co-immunoprecipitation experiments showed that activated CaMKII interacts with Raf-1 in vivo, and that the complex CaMKII/Raf-1 is necessary for ERK activation from different stimuli.[12-14,16] This role of CaMKII in the ERK pathway appears to be a widespread mechanism, as it occurs upon different stimuli (fibronectin-stimulated integrins, serum, insulin, RET/PTC oncogene, Ras^{V12}), in a number of different cell types (thyroid cells, fibroblasts, L6 myotubes, Hep3B, NIH-3T3 and COS-7 cells) with few exceptions (colon adenocarcinoma cells LoVo and prostate cancer cells DU-145, data not shown). Nevertheless, the physiological role of the CaMKII/Raf-1 interplay is likely cell context-dependent, as it participates in and is modulated by the complex crosstalk of signal transduction pathways existing in any living cell. Other studies indicate a link between Ca^{2+}/CaMKII and the MAPK cascade at a site different from Raf-1, at least in some cell types. In Swiss 3T3 cells, calmodulin binds the GTP-bound K-RasB isoform and downmodulates ERK phosphorylation induced by EGF, bombesin, PDGF and serum.[20] In colon adenocarcinoma cells, CaMKII can bind and directly phosphorylate MEK1, resulting in ERK activation, cell cycle progression and cell proliferation.[21] The CaMKII interplay with multiple proteins of the MAPK signaling pathway increases the complexity of the crosstalk between signals and makes the role of this kinase largely cell context-dependent. Another element of complexity is the redundancy of factors that mediate Raf-1 phosphorylation by Ras. Although some authors have argued against a physiological role of Pak family factors in Ras-stimulated Raf-1 activation, this or other factors can

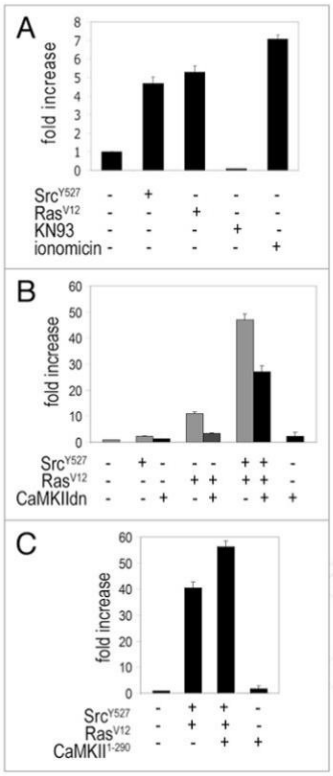

Figure 3. Raf-1 activation by Ras^{V12} is CaMKII mediated in COS-7 cells. (A) CaMKII activity assay. COS-7 cells were transiently transfected with Src^{Y527} or Ras^{V12} and starved from the serum for 24 h. CaMKII activity was determined by in vitro kinase assay. Ionomycin at 2 μM for 10 min and KN93 at 10 μM for 30 min, were used as positive and negative control respectively. All experimental points vs. control $p < 0.01$. (B and C) Raf-1 activity assay. Ras^{V12}, Src^{Y527}, CaMKIIdn and $CaMKII^{1-290}$ were transiently expressed for 48 h in COS-7 cells, where indicated. Raf-1 activity was measured by in vitro kinase assay. The results are presented as fold increase of incorporated cpm. Data are reported as the mean ± standard deviation from duplicate or triplicate experimental points. All differences were significant with the only exception of Src^{Y527} vs. Ras^{V12} + CaMKIIdn in (B) and control vs. $CaMKII^{1-290}$ in (C).

contribute to Raf-1 activity stimulation by certain factors and/or in a cell type-dependent manner.

Our data do not exclude that Pak3 may play the role that was first hypothesized. It is possible that both Pak3 and CaMKII compete for Raf-1 and cooperate to its activation. In inhibition experiments in which Raf-1 activation by Ras^{V12} was determined, a residual Raf-1 stimulation was evident, thus leaving the possibility that other kinases different from CaMKII participate to the modulation of Raf-1 activation.

A major recognized role of Ras is the Raf-1 translocation to the plasma membrane, where the kinase is activated. When Raf-1 was prevented from associating with the plasma membrane by introduction of a mutation, S338 phosphorylation was also prevented, suggesting that S338 phosphorylation takes place at the plasma membrane.[8,22] Most of the studies on CaMKII subcellular localization have been performed in neuronal cells, where it is an important regulator of neuroplasticity. CaMKIIα and β subunits in the postsynaptic fraction prepared from rat forebrains are localized both in the cytosol and in the lipid raft fractions.[23] Also recombinant CaMKIIα expressed in cultured neurons and in COS-7 cells displayed cytosol and plasma membrane localization.[24] In mouse cardiomyocytes, CaMKIIδ subcellular localization is not exclusive, being recognized also in sarcoplasmic reticulum/membrane and nuclear fractions.[25] We have no evidence as to whether the interaction CaMKII/Raf-1 occurs in the cytosol or at the plasma membrane and whether Raf-1 phosphorylation at S338 by CaMKII is restricted to the plasma membrane; however, co-immunoprecipitation studies of fractionated cellular compartments indicate the presence of the Raf-1/CaMKII complex both in the cytosol and with the plasma membrane (not shown).

Raf-1 and B-Raf proteins can interact in several different cell types, forming heterodimers that are mitogen regulated and enhanced by 14-3-3 proteins.[26,27] In HEK293 cells, Ras induces Raf-1/B-Raf complex formation, a mechanism that is involved in the cellular response to growth factor signals.[28] Our previous results did not support a significant role for B-Raf in the signal generated by FN-integrin binding in thyroid cells.[13] Experiments shown in **Figure 3B and C** were replicated, and B-Raf activation was determined by kinase assay. Neither a reduction of Ras^{V12}-stimulated B-Raf activation by CaMKII inhibition, nor B-Raf activity stimulation by active CaMKII was observed (data not shown). These data indicate a Raf isoform selective regulation of Ca^{2+}/CaMKII signaling.

Despite its Raf isoform specificity, CaMKII may have a role in the treatment of tumors acquiring a resistance to B-Raf inhibitors. Selective B-Raf inhibitors potently block cell proliferation and induce apoptosis in B-Raf mutant cancer cells. As a result, several B-Raf inhibitors are currently in clinical development. Increased Raf-1 activity, by Raf-1 overexpression or activation of upstream signaling, has been identified as a mechanism of resistance to the B-Raf inhibitors in pre-clinical studies.[29,30] In these models, tumor cells appear to have switched their dependency from B-Raf to Raf-1, thereby becoming sensitive to Raf-1 inhibitors.[29] In this context, CaMKII inhibitors might overcome resistance to Raf inhibition in a subset of B-Raf mutant tumors.

By modulating Raf-1 activity, CaMKII becomes a component of many signaling pathways with effects on proliferation, apoptosis and differentiation. Inhibitors targeting the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascades have potential application in altered metabolism, cellular senescence, proliferative diseases and prevention of aging.[31,32] The modulation of these pathways by CaMKII inhibitors could represent a therapeutic strategy worthy to be investigated.

In conclusion, our data point to CaMKII being an important component of the Ras/Raf-1/MEK/ERK signal. This kinase

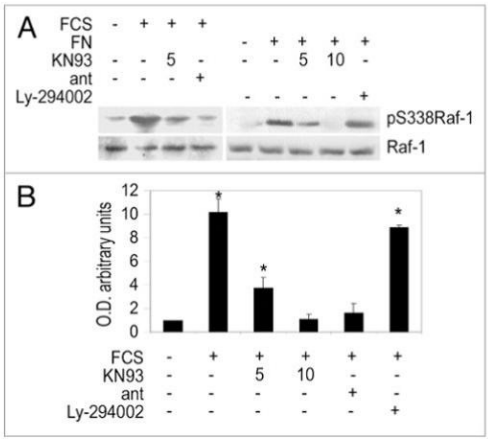

Figure 4. Phosphorylation of Raf-1 at Ser 338 by FCS and FN is CaMKII mediated in TAD-2 cells. (A) TAD-2 cells were starved from serum for 24 h, stimulated for 30 min with FCS or FN, upon 30 min pre-treatment with CaMKII inhibitors, KN93 (5 or 10 or μM) or antCaNtide (5 μM) or with the PI3-K inhibitor Ly-294002 (10 μM). Raf-1 was immunoprecipitated from the cells and pS338Raf-1 and total Raf-1 analyzed by western blot using specific antibodies. (B) Averages and SD of relative expressions of pS338Raf-1 were determined by scanning densitometry of three independent immunoblots. A value of 1 OD arbitrary unit was assigned to the unstimulated sample. *Experimental point vs. unstimulated point $p < 0.01$. The PI3K inhibitory effect of Ly-294002 was confirmed by inhibition of Akt phosphorylation (not shown).

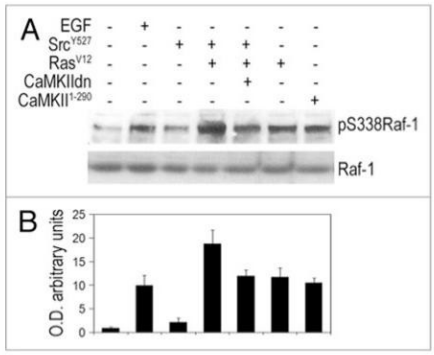

Figure 5. Phosphorylation of Raf-1 at S338 by Ras^{V12} is CaMKII mediated in COS-7 cells. COS-7 cells were transiently transfected with Ras^{V12}, Src^{Y527}, CaMKIIdn, CaMKII$^{1-290}$ for 48 h or treated with EGF 10 ng/mL for 10 min. Raf-1 was immunoprecipitated from the cells and pS338 and total Raf-1 analyzed by western blot using specific antibodies. (A) immunoblot; (B) averages and SD of relative expressions of pS338Raf-1 determined by scanning densitometry of three independent immunoblots. A value of 1 OD arbitrary unit was assigned to the unstimulated sample. All experimental points vs. unstimulated point $p < 0.01$.

phosphorylates Raf-1 at S338 and it is necessary for Raf-1 full activation by Ras and Src. Ca^{2+}/CaMKII signaling is modulated by a large number of factors, and therefore it can regulate the signal transduction downstream of both receptor and oncogenic tyrosine kinases, leading to activation of ERK.

Materials and Methods

Cell cultures and preparation of fibronectin-coated plates. NIH-3T3 fibroblasts, immortalized human fetal thyroid TAD-2 cells (generously donated by Dr. T.F. Davies, Mount Sinai Hospital) and COS-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Inc.,) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine at 37°C in of 5% CO_2 atmosphere. Where indicated, cells were serum starved for 24 h in DMEM, 0.5% BSA (Sigma). To obtain fibronectin (FN) coating, cell culture plates were filled with 100 μg/mL FN (Collaborative Research) in PBS. After overnight incubation at 4°C, FN was removed and the plates were washed with PBS and stored at 4°C.

Expression vectors and transfections. NIH-3T3 and COS-7 cells were transiently or stably transfected with Lipofectamine 2000 according to the manufacturer's recommendations (Gibco Invitrogen). The cDNAs encoding oncogenic Ras isoforms, HRas^{V12} and KRas^{V12}, were subcloned into expression vector pBABE and pcDNA3 respectively. Activated Src mutant, (Src^{Y527}, gently gift from R. Marais, Institute of Cancer Research), was subcloned into expression vector pEF. Rat CaMKIIα kinase-deficient mutant CaMKIIαK42M (CaMKIIdn) was subcloned into pSP72. CaMKII$^{1-290}$ is a truncated sequence of CaMKII that makes the enzyme constitutively active. Both the vectors CaMKIIdn and CaMKII$^{1-290}$ are a generous gift from A.R. Means (Duke University). For transient transfections, the cells were lysed after 48 h. For stable transfections, the cells were cultured for 3 weeks with G418 or puromycine, then single clones were picked-up, screened for expression of the transgene and amplified individually in DMEM 10% FCS supplemented with the appropriate antibiotic.

Western blot and immunoprecipitation. For western blot analysis, cells were washed in PBS buffer and lysed on ice for 30 min in RIPA buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM PMSF, 5 ug/mL leupeptin, 5 ug/mL pepstatin). The lysates were quantified by Biorad DC protein assay and an equal amount of proteins from each sample was diluted with Laemmli buffer. Proteins were resolved by SDS-PAGE and transferred to an Immobilion P membrane (Millipore Corporation). Membranes were blocked by incubation with PBS 0,2% tween, 5% non-fat dry milk for 1 h at room temperature. The membranes were then incubated overnight with primary antibodies at 4°C (dilution 1:1,000), washed for 45 min with PBS 0, 2% tween and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibodies (dilution 1:2,000). Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Bioscience). Computer-acquired images were quantified using ImageJ 1.39 u, National Institutes of Health.

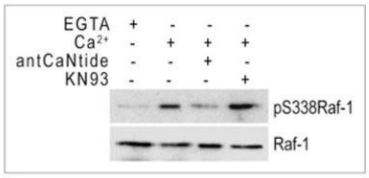

Figure 6. CaMKII directly binds and phosphorylates Raf-1 at S338 in vitro. Raf-1 was immunoprecipitated from unstimulated TAD-2 cells, incubated for 30 min with active CaMKII in the absence (EGTA) or in the presence (Ca^{2+}) of calcium with or without antCaNtide 5 μM or KN93 10 μM pretreatment for 30 min. pS338Raf-1 and total Raf-1 were visualized by western blot.

For the immunoprecipitation procedures, the cells were lysed in immunoprecipitation buffer (50 mM TRIS-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate and 1 mM phenylmethylsulfonylfluoride). Primary polyclonal antibody against all CaMKII isoforms (Santa Cruz Biotechnology) or purified mouse antibody against c-Raf (BD Bioscences PharMingen) was incubated with the lysate for 1 h at 4°C. Successively, Protein G plus/protein A agarose beads (Oncogene Science) were incubated with the immunocomplexes for 2 h at 4°C and used to immunoprecipitate corresponding proteins from 1 mg of total lysate. Mouse monoclonal antibodies to phospho-ERK1/2, Raf-1 and CaMKII and rabbit polyclonal antibody to total ERK1/2 and B-Raf were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Zymed (Invitrogen). Anti-phospho-Raf-1 (Ser338) rat monoclonal was from Upstate Biothechnology.

CaMKII activity assay and inhibitors. CaMKII activity assay was performed as described. In a first reaction step, immunoprecipitated CaMKII was incubated for 30 min at 30°C with 5 mM $CaCl_2$ and 5 μM calmodulin in 50 μl reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 2 μM CaM, 100 nM microcystin, 0.5 mM cold ATP. A 10 μl aliquot from the first reaction was than incubated with 25 mM EGTA, 0.5 mM Autocamtide[33] and 50 μM ATP [1,500 cpm/pmol (γ-^{32}P)ATP] in order to determine CaMKII activity on its peptide substrate Autocamtide. The reaction was performed for 30 min at 30°C and 20 μl aliquots of the reaction mixture were spotted on p81 phosphocellulose filters (Upstate). The level of [^{32}P] incorporation into Autocamtide was determined by liquid scintillation counting. Purified CaM and Autocamtide were a kind gift from A.R. Means (Duke University). Ionomycin (Sigma) was used as positive control of CaMKII activation.

The CaMKs inhibitor KN93, a selective and cell permeating pharmacological inhibitor of the CaMKs (IC_{50} = 370 nM), was from Sigma. The CaMKII-specific peptide inhibitor antCaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN[34] and was made cell permeable by the N-terminal addition of an Antennapedia-derived sequence (antCaNtide: RQI KIW FQN RRM KWK KRP PKL GQI GRS KRV VIE DDR IDD VLK). Calmodulin inhibitors, trifluoperazine (TFP) and N-(6–123 aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7), PI3-K inhibitor (Ly-294002) and Ras farnesylation inhibitor (lovastatin) were from Sigma.

Raf-1 activity assay. Raf-1 activity was evaluated by Raf-1 immunoprecipitation-kinase cascade assay kit (Upstate Biotechnology). Briefly, Raf-1 was immunoprecipitated from 1 mg of cell extracts. The immunocomplexes were washed and incubated with MEK-1 inactive (0.4 μg/assay) and ERK-2 inactive (1 μg/assay) for 30 min at 30°C in 50 μl of reaction mixture ADBI (consisting of 20 mM MOPS pH 7.2, 25 mM β glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol), in the presence of magnesium/ATP. An aliquot of the mixture was then incubated with 20 μg of myelin basic protein (MBP) in the presence of [γ-^{32}P]ATP for 10 min at 30°C and 20 μl aliquots of the reaction mixture were spotted on p81 phosphocellulose filters (Upstate). The level of [^{32}P] incorporation into MBP was determined by liquid scintillation counting.

In vitro phosphorylation of Raf-1 at S338. Raf-1 was immunoprecipitated from 1 mg of cells extract. The immunocomplexes and active recombinant CaMKII were incubated in the presence of EGTA or calcium for 30 min at 30°C in 50 μl of reaction mixture ADBI. The reaction was quenched with Laemmli buffer; proteins were separated by SDS-PAGE through a 10% polyacrylamide gel, transferred to an Immobilion P membrane and phosphorylation visualized by phosphospecific antibody.

Statistical analysis. Results are presented as the mean ± SD. Statistical analysis was performed by using the t-test student. The level of significance was set at p less than 0.01.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

Radioiodide induces apoptosis in human thyroid tissue in culture

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Abstract Radioiodide (^{131}I) is routinely used for the treatment of toxic adenoma, Graves' disease, and for ablation of thyroid remnant after thyroidectomy in patients with thyroid cancer. The toxic effects of ionizing radiations on living cells can be mediated by a necrotic and/or apoptotic process. The involvement of apoptosis in radiation-induced cell death in the thyrocytes has been questioned. The knowledge of the mechanisms that underlie the thyrocyte death in response to radiations can help to achieve a successful treatment with the lowest ^{131}I dose. We developed a method to study the effects of ^{131}I in human thyroid tissue in culture, by which we demonstrated that ^{131}I induces thyroid cell apoptosis. Human thyroid tissues of about 1 mm^3 were cultured in vitro and cell viability was determined up to 3 weeks by the MTT assay. Radioiodide added to the culture medium was actively taken up by the tissues. The occurrence of apoptosis in the thyrocytes was assessed by measuring the production of a caspase-cleavage fragment of cytokeratin 18 (M30) by an enzyme-linked immunoassay. Neither variation of cell number nor spontaneous apoptosis was revealed after 1 week of culture. ^{131}I added to the culture medium induced a dose-dependent and a time-dependent generation of M30 fragment. The apoptotic process was confirmed by the generation of caspase-3 and PARP cleavage products. These results demonstrate that ^{131}I induces apoptosis in human thyrocytes. Human thyroid tissue cultures may be useful to investigate the cell death pathways induced by ^{131}I.

Keywords Radioiodide therapy · Apoptosis · Thyroid

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Introduction

Radioiodide (^{131}I) treatment is commonly used for ablation of thyroid remnant after thyroidectomy in patients with thyroid cancer, in patients with toxic adenoma and Graves' disease and may represent an alternative to surgery in nodular goiter [1–5]. The rationale for the radioiodide ablation of the thyroid remnant after thyroidectomy in patients with differentiated thyroid cancer is to enable the follow-up by serum thyroglobulin measurement. Although the results are inconsistent between studies and there is no direct evidence, some observational evidence suggest that radioiodide ablation with ^{131}I may reduce the risk of recurrence, development of metastases, and long-term mortality from differentiated thyroid cancer [6]. In patients with toxic adenoma or Graves disease, radioiodide treatment is a useful

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option alternative to surgery. Although many centers administer a standard activity of ^{131}I (100 mCi, 3.7×10^9 Bq) for ablation of thyroid remnant after thyroidectomy in patients with differentiated thyroid cancer and a tailored dose for toxic adenoma and Graves disease, there is uncertainty over the lowest effective activity of radioiodide that can achieve successful treatment. For this reason, the mechanisms by which ^{131}I exerts its cytotoxic effects must be studied more in depth. Excess of iodide has a toxic effect on thyroid cells [7, 8]. However, the amount administered is minimal and its cytotoxicity is entirely due to the thyroid cell exposure to ionizing radiations. Cell toxicity induced by ionizing irradiation is usually attributed to DNA damage to target cells, thus triggering cell death by apoptosis and/or necrosis. DNA damage from radiation occurs either by a direct effect or indirectly through the formation of free radicals [9]. Multiple signaling pathways are activated in mammalian cells by DNA-damaging agents, leading to altered gene regulation, some of them involved in DNA repair, others representing a more generalized response to injury [10]. Besides the DNA damage, ionizing radiations damage other cellular compartments through the production of reactive oxygen species and lipid peroxidation of the plasma membrane. Impairment of cellular compartments and reactive oxygen species can induce cell death also by necrosis. Following ^{131}I administration to rats, both apoptosis and necrosis findings were observed [11]. However, other experimental evidence suggested that ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells [12]. In the same study, neither exposure of human thyroid cells in vitro to ionizing radiation nor thyroid gland irradiation in the rat induced thyroid cell apoptosis. More recent data suggest that the apoptotic or necrotic nature of the cytotoxic effect of ^{131}I is dose dependent [13]. It has been supposed that high ^{131}I doses produce mainly necrotic phenomena, whereas low ^{131}I activity induces apoptosis. Hershman et al. recently developed an in vitro model in rat thyroid cells by which it is possible to study molecular aspects of the cellular damage induced by ^{131}I [14]. We developed a method to study the effects of ^{131}I in human thyroid tissue in culture, by which we demonstrated that ^{131}I induces thyroid cell apoptosis.

Materials and methods

Thyroid cell cultures

Thyroid tissues were obtained from controlateral lobe of papillary thyroid cancer undergoing thyroidectomy, after patients gave their written consent. The tissues were cut in small pieces of 1 mm^3, washed in PBS, and cultured in a 5 % CO_2 atmosphere at 37 °C, in F-12 medium supplemented with 10 % FCS and a mixture of thyrotropin (TSH, 10 mU/ml), insulin (10 μg/ml), hydrocortisone (5 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (6H). All tissues were cultured for 1 day before any experiments. Glands with concurrent Hashimoto's thyroiditis were excluded [15]. Seven-day cultured tissues stained with hematoxylin and eosin displayed normal follicular architecture (Fig. 1).

MTT assay

Cell viability and proliferation was determined using the MTT assay [16]. For each experimental point, three tissue fragments from the same gland were put together in the same well of a 24-well plate. Following the designated treatment, the culture medium was removed and 500 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT) (Sigma Chemical Co., St. Louis, MO), 0,5 mg/ml in F-12 medium were added to each well. After 4 h at 37 °C, the medium was removed from each well and replaced with an equal volume of 0.04 m HCl/isopropanol. Following an overnight incubation in darkness, the dissolved MTT crystals were quantitated. Optical densities were obtained using a test wavelength of 550 nm (Dynatech MR5000 microelisa spectrophotometer, Chantilly, VA), and total O.D. normalized per weight of tissue.

Measurement of apoptosis of epithelial cells

To quantify the apoptosis occurring in thyroid epithelial cells, we measured the production of the neo-epitope formed upon caspase-cleavage of cytokeratin 18 at position Asp396 (M30) by an enzyme-linked immunoassay . Caspase-cleaved cytokeratin-18 was measured with M30-

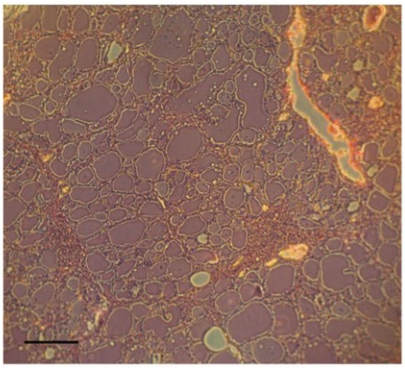

Fig. 1 Microscopic appearance of cultured thyroid tissue. Histological appearance of untreated 7-day cultured tissue, stained with H&E. The tissue shows normal follicular architecture. *Bar line* 50 μm

Apoptosence assay (Peviva, Alexis, San Diego, CA). As for the MTT assay, for each experimental point, 3 tissue fragments from the same gland were put together in the same well of a 24-well plate in 0.5 ml of medium. Following the designated treatment, 50 µl of the culture medium was removed and the assay was performed following the manufacturer protocol. Optical densities were obtained using a test wavelength of 415 nm (Dynatech MR5000), and total O.D. normalized per weight of tissue.

Western blot

Tissues were washed in cold PBS and homogenized with a mortar and pestle in RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate in PBS) supplemented with protease inhibitor phenylmethylsulfonyl fluoride (Sigma). Lysates were centrifuged at 12,000×*g* for 15 min at 4 °C and supernatant was recovered. The protein concentration in tissue lysates was determined by protein assay (Bio-Rad Laboratories, Inc. Richmond, CA), and 50 mg of total protein from each sample was boiled for 5 min in Laemmli sample buffer (125 mm Tris pH 6.8, 5 % glycerol, 2 % SDS, 1 % b-mercaptoethanol, and 0.006 % bromophenol blue). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Hybond-ECL Nitrocellulose, Amersham Pharmacia Biotech, Rainham, UK). Membranes were blocked by 5 % nonfat dry milk, 1 % ovalbumin, 5 % FCS, and 7.5 % glycine; and after three washes, the membranes were incubated for 1 h at 4 °C with 0.5 mg/ml of rabbit polyclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 °C with horseradish peroxidase-conjugated antirabbit secondary antibodies (Bio-Rad Laboratories, Inc.) diluted 1:2,000 in PBS, Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Rabbit polyclonal antibodies to Caspase-3 and PARP were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using the *t* test. The level of significance was set at $p < 0.05$. Bonferroni multiplicity correction was applied to adjusts the threshold for statistical significance.

Results

Thyroid cell viability in tissue cultures

We applied the MTT assay to evaluate the viability of thyroid cells in tissue cultures. Because the thyroid gland is largely perfused by blood vessels, the erythrocytes are abundant in tissue cultures. The mitochondria present in the erythrocytes affects the MTT assay and must be removed. We tested the efficacy of ammonium chloride to lyse the erythrocytes, treating the tissues with a lysis buffer (NH_4Cl–NaH_2CO_3–EDTA) up to 7 h and then performing the MTT assay. The test showed a rapid reduction of the O.D. that achieved a plateau by 2 h of treatment (Fig. 2). On the basis of this result, the subsequent MTT assays were preceded by 2 h treatment with ammonium chloride lysis buffer. To test the cell viability in thyroid tissue cultures, MTT assays were performed up to 3 weeks of culture (Fig. 3). After the first week of culture, no significant change in MTT assay was observed. By 2 weeks, the O.D. increased and only after 3 weeks of culture a significant O.D. increase was demonstrated. These results indicate that small thyroid tissues can be cultured in vitro up to 3 weeks without loss of cell viability.

^{131}I uptake in thyroid tissues cultures

To determine whether the thyroid cells in tissue cultures were still able to trap and concentrate iodide, tissue fragments were cultured for 1 day and then incubated with fresh medium containing 1×10^5 cpm ^{131}I, in the presence of serum and 6H, with or without 1 mM $KClO_4$. After 3–6 h, the tissues were washed and the incorporated radioactivity was counted in a gamma counter (Fig. 4). After 3 h of incubation, $KClO_4$ reduced the incorporated radioactivity although the difference was not significant. By 6 and 12 h, $KClO_4$ significantly affected the incorporation of radioactivity, being respectively 48 and 38 %

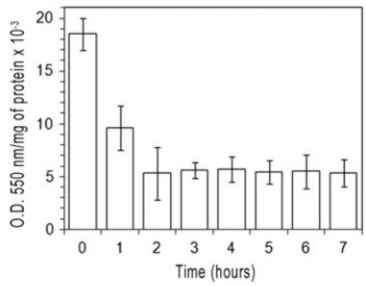

Fig. 2 Interfering effect of erythrocytes on MTT assay in thyroid tissue cultures. One mm^3 thyroid tissue samples were carefully weighted and treated at 37 °C for the indicated time with 1 ml ammonium chloride erythrocyte lysis buffer. Then, the tissues were washed and MTT assay was performed. Data are presented as mean and s.d. of triplicates O.D./mg of thyroid issue. All experimental points versus time 0 were significant after Bonferroni correction, $p < 0.007$ by Student's *t* test

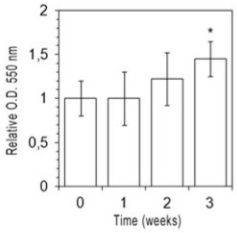

Fig. 3 MTT assay in thyroid tissue cultures. Thyroid tissues of 1 mm^3 were carefully weighted and cultured up to 3 weeks in the presence of serum and 6H. At the indicated time, the tissues were washed, erythrocytes were lysed, and MTT assay was performed. Total O.D./mg of tissue was calculated. Results are reported as mean and s.d. relative O.D. 550 nm of triplicate experiments. $*p = 0.012$, Student's t test

lower. These results indicate that the ability to uptake iodide was retained by thyroid tissues in culture.

Measurement of apoptosis in thyroid tissues

To assess the apoptosis of thyroid cells in cultured tissues, we used an immunometric assay which determines the amount of caspase-3-induced degradation of cytokeratin-18. Thyroid tissues were treated for 24 h with the apoptotic inducer staurosporine, or the necrosis inducer NaN_3 (Fig. 5). A large amount of M30 cytokeratin fragment was generated by the staurosporine treatment, while as expected NaN_3 did not.

Apoptosis induction by ^{131}I in thyroid tissue cultures

Thyroid tissues were cultured for 1 day and then placed in 24-well plates with medium containing ^{131}I. After 1 h, 1, 3,

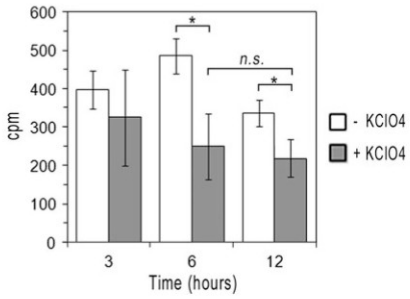

Fig. 4 ^{131}Iodide uptake in thyroid tissues in culture. One-week-old thyroid tissues were cultured up to 12 h in the presence of serum, 6H and 1×10^5 cpm ^{131}I, with or without 1 mM $KClO_4$. At the indicated time, the tissues were washed and incorporated cpm was counted by a gamma counter. $*p < 0.01$, Student's t test; *n.s.* not significant

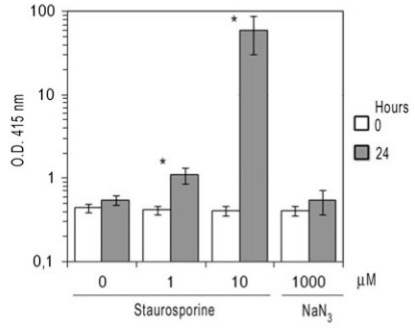

Fig. 5 Assessment of apoptosis in cultured thyroid tissues. Thyroid tissues were treated for 24 h with 0.1–1 µM staurosporine or 1 mM NaN_3. Then, the culture mediums were recovered and the presence of the M30 cytokeratin fragment was assessed by the M30 Apoptosense assay. Results are reported as total O.D. at 415 nM. $*p < 0.01$, Student's t test

or 7 days, an aliquot of medium was removed and the M30 concentration was determined by the M30 Apoptosense assay (Fig. 6). In the absence of ^{131}I, no spontaneous M30 production occurred after 3 days of culture, while it was detectable after 7 days. ^{131}I induced a significant M30 production already by 3 days of culture, demonstrating that ^{131}I induced apoptosis of thyroid epithelial cells. In a parallel experiment, thyroid tissues were cultured for 3 days as above. Then, the tissues were homogenized, proteins were extracted and analyzed by Western blot (Fig. 7). Following the treatment with 3.7×10^6 and 3.7×10^7 Bq ^{131}I, cleaved caspase-3 and PARP fragments were evident, confirming that apoptosis was occurring.

Discussion

Thyroid follicles are three-dimensional structures embedded in extracellular matrix which cannot be retained for a long term in conventional monolayer, floating, or organ cultures, [17, 18]. Thyrocyte interactions with the extracellular matrix components are mediated by integrins and generate intracellular signals that regulates cell physiology [19–21]. Furthermore, the absence of blood flow in tissue culture hampers gas and nutrients diffusion. Thus, we cultured human thyroid tissue in fragments of about 1 mm^3, sufficiently small to allow adequate O_2/CO_2 exchange and metabolites diffusion with culture medium. Cell viability was investigated by the MTT assay, based upon the tetrazole reduction to formazan in the mitochondria of living cells. MTT assay demonstrated that no significant variation of cell viability occurred in the first 2 weeks of culture. By that time, a moderate increase of absorbance was noted,

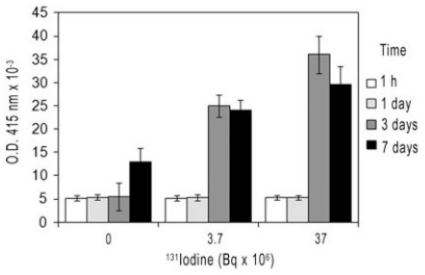

Fig. 6 Induction of apoptosis by ^{131}Iodide in cultured thyroid tissues. Thyroid tissues were cultured up to 7 days in the presence of ^{131}I. At the indicated time, 10 % of culture medium was recovered and the presence of M30 was assessed by the M30 Apoptosense assay. Results are reported as mean O.D. 415 nM of triplicate experiments. 3.7×10^6 and 3.7×10^7 Bq for 3 and 7 days, and no ^{131}I for 7 days versus 1 h, $p < 0.01$; 3.7×10^6 and 3.7×10^7 Bq for 3 and 7 days versus corresponding time without ^{131}I, $p < 0.016$ by Student's t test

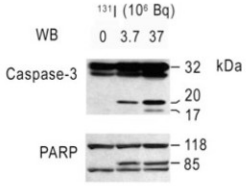

Fig. 7 Western blot analysis for caspase-3 and PARP thyroid tissues were cultured for 3 days in the presence of ^{131}I. Then, the tissues were washed, homogenized, lysed in RIPA buffer and protein extracts were separated by SDS-PAGE with 12 % acrylamide concentration. The proteins were blotted onto nitrocellulose membrane, incubated with polyclonal antibodies to intact and fragmented caspase-3 and PARP proteins and evidenced by ECL system

compatible with a change in mitochondrial dehydrogenase activity or increase of cell number, due to thyrocytes or more likely fibroblasts proliferation. Significant M30 production was detected only after 7 days of culture (Fig. 5), indicating that spontaneous apoptosis was occurring by that time. In light of these results, all the experiments were performed in the first week of culture. The ^{131}I isotope emits γ-rays but it is used for thyroid ablation by virtue of its β-particles emission. Its cell toxicity is primarily the result of the β-particle radiation, which has a path length of 1–2 mm in water. This means that the thyrocytes in tissue cultures were exposed to β-particle radiations coming from the taken up ^{131}I isotope as well as from the ^{131}I isotope present in the culture medium in which tissues were embedded. Regardless the source of β-particle radiations, this model revealed to be suitable to investigate the molecular mechanisms by which ^{131}I kills thyrocytes. High doses of ionizing radiations lead to genetic damage, mutations, and finally cell death. DNA damage from radiation can occur either by a direct effect or indirectly through the formation of free radicals [9, 22]. In both cases, chromosomal damage leads to the activation of the enzymes responsible for DNA repair, cell cycle arrest, or apoptosis. A key protein in this process is p53 which, after radiation exposure, translocates to the nucleus, where it mediates the transcription of specific genes. Although this is a general mechanisms operating in all mammalian cells, the involvement of apoptosis and the role of p53 in radiation-induced cell death in the thyrocytes have been questioned. Namba H et al. demonstrated that p53-WAF1/Cip1 pathway plays a central role in induction of G1 arrest following irradiation in human thyrocytes in culture [23]. Both apoptosis and necrosis findings were observed in the thyroid of rats treated with ^{131}I [11]. At the same time, evidence have been produced in favor of a dose-dependent apoptotic or necrotic cell death, that is high ^{131}I doses can produce mainly necrotic phenomena, whereas low doses induces mainly apoptosis [13]. However, in a following study in human thyroid primary cells in culture and intact rat, exposure to ionizing radiation confirmed that DNA end-jointing activities were promoted by p53 induction in thyroid cells, but failed to demonstrate the induction of apoptosis [12]. The measurement of the M30 fragment enabled us to quantify the apoptotic phenomena involving the thyrocytes as this protein fragment is generated by the enzymatic activity of caspase-3 on cytokeratin 18, expressed in the thyroid gland only by the thyroid follicular cell. The generation of M30 fragment following 3 days of treatment with ^{131}I indicates the occurrence of apoptosis but does not exclude also the occurrence of necrotic phenomena. Apoptosis is an active mechanism involving protein–protein interactions and regulation of gene expression, with a fundamental tumor suppression role [24]. Cancer cells can reduce the pro-apoptotic machinery thus becoming resistant to apoptosis-mediated toxic factors. p53 inactivation or down regulation and NF-kB modulation are just examples of how aberrant apoptotic signaling can contribute to cancer development through resistance to apoptosis [25, 26]. Administration of ^{131}I is currently used in the treatment of differentiated thyroid carcinoma not only to ablate the thyroid remnants following surgery but also to treat the metastatic disease. Aberrant apoptotic signaling in differentiated thyroid cancer metastasis can induce resistance to radiotherapy. Cell–extracellular matrix components interactions are crucial in the normal thyroid follicle architecture as well as in tumor invasion and metastasis [27]. This is even more important when genetic alterations like mutated BRAF or soluble factors (i.e., cytokines) released by infiltrating lymphocytes alters the normal cell polarity and the expression of the sodium iodide symporter [28–30]. This experimental model using normal human thyroid tissue may be useful in investigating the cell

death pathways induced by ^{131}I and identify the mechanisms by which thyroid cancer cells become resistant to radioiodide treatment.

Conflict of interest The authors declare that they have no conflict of interest.

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Interferon-γ inhibits integrin-mediated adhesion to fibronectin and survival signaling in thyroid cells

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Abstract

Hashimoto's thyroiditis is the most frequent autoimmune disorder, characterized by the presence of a large lymphocytic infiltration and secretion of inflammatory cytokines in the thyroid. Infiltrating lymphocytes and cytokines play a pivotal role in the progression of HT, characterized by the progressive destruction of the normal follicular architecture of the gland and death of follicular cells, ending with loss of thyroid function. Integrins are plasma membrane receptors for the cell–extra-cellular matrix components, with both structural and signaling functions. Integrin-mediated fibronectin (FN) binding is necessary for the correct function and survival of thyroid follicular cells. The purpose of this study was to determine the effect of interferon-γ (IFN-γ) stimulation on integrin expression and signaling in the thyroid cell. Cytotoxicity, integrin expression, cell adhesion to FN, and FN-stimulated ERK and AKT phosphorylation were determined in a normal human thyroid cell line treated with IFN-γ. IFN-γ induced apoptosis and reduced the expression of the integrin αvβ3. Integrin-mediated cell adhesion to FN was strongly impaired. Similarly, FN-stimulated ERK and AKT phosphorylation were inhibited. In conclusion, our study in a thyroid cell model demonstrates that IFN-γ induces apoptosis and inhibits the expression of the integrin αvβ3, reducing cell adhesion to FN and the succeeding outside-in signaling. These results suggest that integrins mediate the cytotoxic effect of IFN-γ and are involved in the destructive mechanism of autoimmune thyroiditis.

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Introduction

Chronic lymphocytic thyroiditis, also known as Hashimoto's thyroiditis (HT), is the most common autoimmune disease, with a prevalence of 5–10% in the general population (Tunbridge *et al.* 1977, 1981). Auto-antibodies to thyroid antigens and lymphocyte infiltration on histology are the hallmark of HT (Stassi *et al.* 2000, Caturegli *et al.* 2007). A transient modest hyperthyroidism and a small goiter can be the first clinical findings of this disease until the gradual loss of thyroid function appears with hypothyroidism. The reduced thyroid function is due to the progressive destruction of the normal follicular architecture of the gland and death of follicular cells. T helper type 1 (Th1) is the main subset of lymphocytes present in HT and has a crucial role in the pathogenesis of the disease by secreting inflammatory cytokines such as interferon-γ (IFN-γ; Salgame *et al.* 1991, Romagnani 1994, Carter & Dutton 1996, Pala *et al.* 2000, Mazziotti *et al.* 2003, Santaguida *et al.* 2011). The role of IFN-γ in the pathogenesis of thyroid damage in HT is supported by the evidence of intra-thyroidal cytokine secretion and by experimental evidences in cells in culture and in animal models (Liblau *et al.* 1995, Roura-Mir *et al.* 1997, 2005, Stassi *et al.* 2000, 2001).

Integrins are a group of cell-surface heterodimers characterized by a common β1 chain non-covalently associated with a distinctive α subunit (Hemler *et al.* 1987). The members of this family are receptors for the components of the extra-cellular matrix (ECM), involved in cell–ECM interactions. Their level of expression undergoes quantitative and qualitative changes upon differentiation, neoplastic transformation, and hormone stimulation (Dedhar 1989, Heino & Massague 1989, Plantefaber & Hynes 1989, Wilkins *et al.* 1991, Vitale *et al.* 1994, 1995). The αβ integrin complex binds ECM by its extracellular domain and interacts with cytoskeletal proteins by its intracellular domain, contributing to the stability of the tissue architecture (Otey *et al.* 1990). Some integrins are components of a subcellular structure called focal adhesion, where they interplay with regulatory proteins (Luna & Hitt 1992). Integrin binding to the ECM generates multiple intracellular signals that contribute to the regulation of many cell processes, including differentiation,

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growth, and survival (Mortarini *et al.* 1992, Pasqualini & Hemler 1994). The integrin expression profile is cell-type-dependent and is modulated by a number of factors, including cell-to-cell contact, extracellular soluble factors, and tumor transformation. Normal thyroid cells have a limited integrin expression repertoire (α3β1 and αvβ3) that changes upon cell-to-cell contact and tumor transformation (Vitale *et al.* 1993, 1994, 1995, Illario *et al.* 2003). Integrin activation by fibronectin (FN) has important biological effects in the thyroid cell, regulating cytoskeletal organization and stimulating cell proliferation and survival (Vitale *et al.* 1998, Di Matola *et al.* 2000). The aim of this study was to investigate whether integrins mediate the cytotoxic effect exerted by IFN-γ on thyroid cells. For this purpose, we investigated the effect of IFN-γ on cytotoxicity, integrin expression, adhesion to FN, and FN-stimulated signaling in a human thyroid cell model. We found that INF-γ reduces the expression of the integrin αvβ3 and strongly inhibits the FN-dependent intracellular signalings, modulating cell proliferation and survival.

Materials and Methods

Cell cultures

The TAD2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells, was generously donated by Dr T F Davies (Mount Sinai Hospital, New York, NY, USA) and cultured in a 5% CO_2 atmosphere at 37 °C in DMEM (4·5 g glucose) and 10% FCS. The TAD2 cell line has the same integrin expression profile and FN-stimulated signaling of normal human thyroid cells in culture (Vitale *et al.* 1997, Illario *et al.* 2003). Medium was changed every 3–4 days. Cells were detached by 0·5 mm EDTA in calcium- and magnesium-free PBS with 0·05% trypsin. When needed, the cells were serum starved in 0·5% BSA and DMEM for 12–18 h before stimulation. To obtain a FN or BSA coating, cell culture plates were filled with the appropriate FN (Collaborative Research, Bedford, MA, USA) or BSA (Sigma) dilution in PBS. After overnight incubation at 4 °C, FN was removed, and the plates were washed with PBS and stored at 4 °C.

Antibodies to integrins and flow cytometric analysis

All the antibodies specific for single integrin subunits or heterodimers were mouse monoclonals purchased from Santa Cruz Biotechnology, Inc. Fluorescein-conjugated anti-mouse IgG was purchased from Ortho (Raritan, NJ, USA). Cells harvested from cell cultures by trypsin/PBS were incubated with MABs for 1 h at 4 °C in PBS and 0·5% BSA (BSA/PBS), washed in the same buffer, and incubated again with the second fluorescein-conjugated antibody for 30 min at 4 °C. Cells were resuspended in BSA/PBS and analyzed by flow cytometry. Single cell suspensions were analyzed by a FACScan (Becton Dickinson, Mountain View, CA, USA). Forward scatter vs side scatter analysis was performed on a logarithmic scale using a high forward threshold to cut off cellular and collagen debris. Cytofluorimetric estimation of DNA cell content was performed as described (Illario *et al.* 2003). Floating cells were collected, washed in cold PBS, added to adherent cells, and trypsinized. Cells were washed again in PBS and fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes, and cells were incubated in PBS, 50 μg/ml propidium iodide, 10 μg/ml ribonuclease A, and deoxyribonuclease-free overnight at 4 °C. Cells were then analyzed by flow cytometry using a FACScan.

Cell attachment assay

The assay was performed in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA). The wells were filled with 100 ml of the appropriate dilution in PBS of FN (Collaborative Research). After overnight incubation at 4 °C, the plates were washed with PBS, filled with 100 ml 1% heat-denatured BSA, and incubated for 1 h at room temperature. Then, plates were washed and filled again with 100 ml/well PBS, 0·9 mM $CaCl_2$, and 0·5 mM $MgCl_2$ containing 2×10^4 cells. After 30 min at 37 °C, plates were gently washed three times with PBS, and the attached cells were fixed with 3% paraformaldehyde for 10 min followed by 2% methanol for 10 min and finally stained with 0·5% crystal violet in 20% methanol. After 10 min, plates were washed with tap water, the stain was eluted with a solution of 0·1 M sodium citrate, pH 4·2, in 50% ethanol, and the absorbance at 540 nm was measured by a spectrophotometer. Where indicated, the cells were coincubated with 500 μg/ml of integrin/FN-binding inhibitory peptide RGSP (Gly-Arg-Gly-Asp-Ser-Pro) or the ineffective peptide RGE (Gly-Arg-Gly-Glu-Ser-Pro), Telios (San Diego, CA, USA). All experiments were performed in quadruplicate.

Western blot

The cells were lysed in Laemmli buffer (125 mM Tris (pH 6·8), 5% glycerol, 2% SDS, 1% 2β-mercaptoethanol, and 0·006% bromophenol blue) and resolved by SDS–PAGE. Proteins were then transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp., Bedford, MA, USA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7·5% glycine, and after three washes, the membranes were incubated for 1 h at 4 °C with mouse monoclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 °C with a HRP-conjugated anti-mouse secondary antibody. After a final wash, protein bands were detected by an ECL system (Amersham Pharmacia Biotech). Mouse MABs to ERK and phosphorylated ERK, Akt and phospho-threonine-308-Akt, caspase-3, and PARP were from Santa Cruz Biotechnology, Inc.

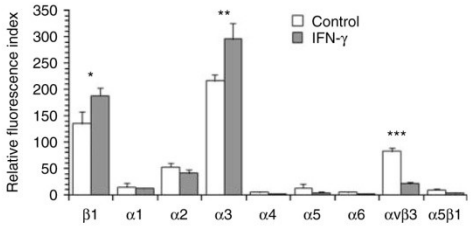


Figure 1 IFN-γ modulates integrin expression. TAD2 cells cultured at low confluence were treated for 3 days with IFN-γ (10 ng/ml), harvested by mild trypsinization, and incubated with MABs specific for single integrin subunits (β1, α1, α2, α3, α4, α5, and α6) or whole receptors (α5β1 and αvβ3) followed by the secondary fluorescein-conjugated antibody. The relative fluorescences were measured by flow cytometry as described in Materials and Methods section. The expression of each integrin etherodimer or single subunit is reported as relative fluorescence index: mean of experimental mean fluorescence/control mean fluorescence of three independent primary cultures. *P=0·048; **P=0·039; ***P=0·0017 by Student's t-test.

Statistical analysis

Results are presented as the mean ± S.D. Statistical analysis was performed using the Student's t-test or by two-way ANOVA. The level of significance was set at $P<0·05$. Statistical analyses were conducted using SPSS 18.0 (IBM Corporation).

Results

INF-γ modulates the expression of FN receptors of the integrin family in TAD2 cells

All the experiments were performed with TAD2 cells cultured at 75% confluence. The cells were treated for 3 days with IFN-γ, then integrin expression was assessed by flow cytometry with specific antibodies for the subunits β1, α1, α2, α3, α4, α5, α6, or the heterodimers αvβ3 and α5β1 (Fig. 1). The expression of the α3 and β1 subunits was increased by INF-γ treatment. More robust was the effect of INF-γ on the αvβ3 heterodimer, whose expression was reduced by 76·6%.

IFN-γ inhibits thyroid cell adhesion to FN

To investigate whether the adhesion of thyroid cells to FN was changed by IFN-γ, TAD2 cells were treated with the cytokine for 3 days and a cell attachment assay was performed (Fig. 2). Cell adhesion in the presence of the RGDSP peptide, a specific inhibitor of integrin binding to FN, was reduced by 84·7%, demonstrating the major role of integrin receptors in the adhesion to immobilized FN. IFN-γ treated cells showed a remarkable reduction of attachment to FN. The reduction of integrin-dependent attachment (CTRL+RGE−CTRL+GRDSP/IFN-γ+RGE−CTRL+GRDSP) in 10 and 50 μg/ml FN adhesion assays was 71 and 65% respectively. Two-way ANOVA assessment of effects of IFN-γ indicated a significant difference between IFN-γ+RGE and CTRL+RGE (F-ratio=5·38, P=0·045), a significant difference among FN concentrations (F-ratio=10·78, P=0·009), and a significant interaction (F-ratio=17·7, $P<0·001$). Moreover, two-way ANOVA for comparison of RGE to RGDSP indicated a significant difference between CTRL+RGE and CTRL+RGDSP (F-ratio=10·73, P=0·010), a significant difference among FN concentrations (F-ratio=4·88, P=0·049), and a significant interaction (F-ratio=17·7, $P<0·001$). This data are consistent with the reduction of αvβ3 expression and/or reduced affinity of α3β1 to FN induced by IFN-γ.

Effect of IFN-γ on the cell cycle and survival

The effect of IFN-γ on the cell cycle was evaluated in TAD2 by flow cytometry analysis. TAD2 cells were plated onto FN-coated plates and cultured for 3 days in the presence of IFN-γ or left untreated. Then, all the cells, both floating in the medium and adherent, were collected, stained with propidium iodide, and analyzed by FACScan (Fig. 3A). The number of cells in the G0–G1 phase decreased from 49% in untreated cells to 38 and 34% after 10 and 100 ng/ml IFN-γ treatment respectively. The number of hypodiploid cells

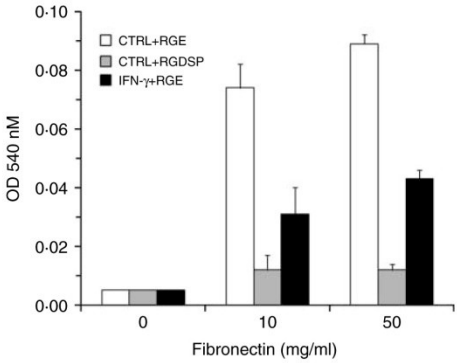
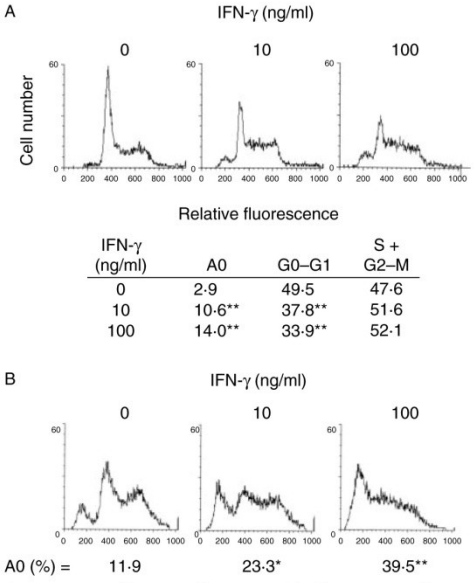


Figure 2 Effect of IFN-γ on thyroid cell adhesion to FN. Ninety-six-well microtiter plates were coated with soluble FN at 10 or 50 μg/ml and saturated with heat denatured BSA. TAD2 cells were untreated (CTRL) or treated with IFN-γ, 10 ng/ml for 3 days. A total of 2×10^4 thyroid cells were seeded in the wells, with RGE or RGDSP peptides, and the plates were incubated at 37 °C for 30 min. Attached cells were measured as described in Materials and Methods section. Data are reported as the mean ± S.D. of quadruplicate experiments. RGE and RGDSP, 500 μg/ml. Two-way ANOVA analysis for IFN-γ: IFN-γ+RGE vs CTRL+RGE (F-ratio=5·38, P=0·045), among FN concentrations (F-ratio=10·78, P=0·009), interaction (F-ratio=17·7, $P<0·001$). Two-way ANOVA for comparison of RGE to RGDSP: CTRL+RGE vs CTRL+RGDSP (F-ratio=10·73, P=0·010), among FN concentrations (F-ratio=4·88, P=0·049), interaction (F-ratio=17·7, $P<0·001$).



IFN-γ (ng/ml)	A0	G0–G1	S + G2–M
0	2·9	49·5	47·6
10	10·6**	37·8**	51·6
100	14·0**	33·9**	52·1

Figure 3 DNA cell content of IFN-γ treated cells. (A) TAD2 cells were plated onto 10 μg/ml FN-coated plates and cultured for 3 days in the presence of 10 or 100 ng/ml IFN-γ or left untreated. Then, both adherent and suspended cells were collected, stained with propidium iodide, and analyzed by FACScan. (B) The cells were treated as in (A) for 6 days. S.D.s were <15%. A0, hypodiploid cells. Experimental point vs 0 point: *$P<0{\cdot}05$, **$P<0{\cdot}005$ by Student's *t*-test.

increased from 2·9 to 11% and 14%. The presence of a large number of DNA-loosing cells prevented distinguishment between S and G2–M phases, which appeared merged. By 6 days of treatment, the effect of IFN-γ was massive, about 20% of the cells were detached and up to 39·5% of the cells (both floating and adherent) were hypodiploid (Fig. 3B). These data demonstrate a time- and concentration-dependent cytotoxicity of IFN-γ.

IFN-γ induces apoptosis in thyroid cells

Caspase-3 cleavage and PARP fragmentation were investigated by western blot to assess the occurrence of apoptosis (Fig. 4). TAD2 cells were treated for 3 days with IFN-γ and analyzed, demonstrating that the hypodiploid cells were due to loss of fragmented DNA following apoptosis.

Effect of IFN-γ on FN-induced signaling

To determine whether IFN-γ affects the Ras/Raf/MEK/ERK and phosphatidyl-inositol 3 kinase (PI3K)/AKT signaling, thyroid cells were untreated or treated with IFN-γ for 3 days, starved from serum overnight, and plated onto immobilized BSA or FN. ERK phosphorylation was achieved by serum stimulation. After 30 min, the cells were lysed and phosphorylated Akt or ERK were evidenced by western blot (Fig. 5). Adhesion to FN stimulated both Akt and ERK phosphorylation. The treatment with IFN-γ strongly inhibited FN-induced phosphorylation of both kinases.

Discussion

IFN-γ, TNF-α, and IL2 can modulate the expression of β1 integrins, and therefore change the cell–ECM interaction in some cell types including fibroblasts, endothelial cells, and lymphoid cells (Pirila & Heino 1996). It has been proposed that adhesion molecules are involved in the maintenance of autoimmunity. Some cytokines induce upregulation of α1β1, enhancing the persistence of inflammatory cells in the extralymphatic tissues and the in-site production of cytokines (Ben-Horin & Bank 2004). However, the effects of inflammatory cytokines on thyroid integrin expression and function remained poorly investigated (Marazuela *et al.* 1997). Our study demonstrates that IFN-γ has a cytotoxic effect and modulates integrin expression and function in the TAD2 cell line. This cell line was demonstrated to be a reliable model to study cell–ECM interactions as it has the same integrin expression profile and FN-stimulated signaling of normal human thyroid cells in culture (Vitale *et al.* 1997, 1998, Illario *et al.* 2003). Indeed, some of the experiments of this study have been performed with identical results in thyroid primary cultures (Russo *et al.* 2012). All the experiments were performed with TAD2 cells cultured at 75% confluence to normalize integrin expression, which is downregulated by cell-to-cell contact (Vitale *et al.* 1995). Thyroid cells are polarized epithelial cells, whose basal plasma membrane adheres to ECM components of the basal membrane.

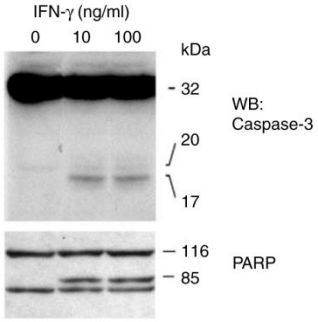


Figure 4 Western blot analysis of caspase-3 and PARP in IFN-γ treated cells. TAD2 cells were plated onto 10 μg/ml FN-coated plates and cultured for 3 days in the presence of 10 or 100 ng/ml IFN-γ or left untreated. Cell lysates were analyzed by western blot with antibodies to caspase-3 and PARP.

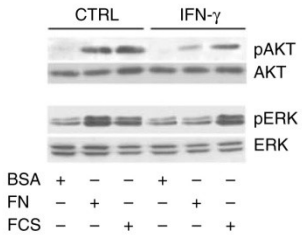


Figure 5 Stimulation of AKT and ERK phosphorylation by adhesion to FN. TAD2 cells were untreated or treated with IFN-γ 10 ng/ml for 3 days. The cells were starved from serum overnight, harvested by mild trypsinization, and seeded in serum-free medium onto BSA-coated plates (BSA) or 50 μg/ml FN-coated plates (FN), or untreated plates in the presence of 10% FCS for 30 min. The cells were lysed and the proteins were analyzed by western blot with antibodies anti-total (AKT and ERK) or anti-phosphorylated AKT ERK (pAKT and pERK).

Impaired adhesion to FN modifies outside-in signaling, inhibits cell proliferation, and triggers a programed cell death known as 'anoikis' (Vitale *et al.* 1997, 1998, 1999, Di Matola *et al.* 2000, Illario *et al.* 2003, 2005). The adhesion assays, with the highly specific integrin/FN-binding inhibitor RGDSP, demonstrated that thyroid cell adhesion to FN was almost exclusively mediated by β1 integrins. Therefore, α3β1 and αvβ3, the only β1 integrins expressed, are the adhesion molecules involved in thyroid cell adhesion to FN. While α3β1 is localized on the entire plasma membrane facing the basal membrane and has mainly a structural function, αvβ3 is localized in the focal adhesions and mediates FN outside-in signaling. Inhibition of FN/αvβ3 binding and the succeeding signaling have important consequence for the cell, including inhibition of proliferation and induction of apoptosis. IFN-γ induced a modest upregulation of α3β1 expression and a more evident downregulation of αvβ3 in TAD2 cells. The remarkably impaired adhesion to FN might be due to the more relevant reduction of αvβ3/FN interaction. However it cannot be excluded that IFN-γ possibly reduced the binding affinity of one or both the receptors. Indeed, the affinity of integrin receptors for their ligands is cell-type-dependent and modulated by different factors (Wilkins *et al.* 1991). The cytotoxic effect of IFN-γ was an apoptotic process, as demonstrated by the caspase-3 and PARP cleavage. This cytokine generates a pleiotropic signaling with multiple effects. It has been previously demonstrated that impairment of αvβ3 adhesion to FN induces apoptosis in thyroid cells (Vitale *et al.* 1998, Illario *et al.* 2003). Although a direct involvement of this integrin in the cytotoxic effect of IFN-γ was not demonstrated in our study, it can be speculated that the inhibition of MAPK and PI3K/AKT signaling is involved in the apoptotic process induced by this cytokine. Indeed, the inhibition of the AKT survival pathway, the same of many growth factors necessary for cell survival, makes the thyroid cell more sensitive to damaging agents and thus might contribute to the cytotoxicity of IFN-γ or of other damaging mechanisms active in the HT. In conclusion, our study in a thyroid cell model demonstrates that IFN-γ induces apoptosis and inhibits the expression of the integrin αvβ3, reducing cell adhesion to FN and the succeeding outside-in signaling. These results suggest that integrins mediate the cytotoxic effect of IFN-γ and play a role in the destructive mechanism of autoimmune thyroiditis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Interferon-γ inhibits integrin-mediated ERK activation stimulated by fibronectin binding in thyroid cells

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Running Head: IFN-γ and integrins in thyroid cells

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ABSTRACT

Hashimoto's thyroiditis (HT) is an autoimmune disorder characterized by the presence of specific antibodies and by a lymphocytic infiltration of the thyroid secreting inflammatory cytokines. Macrophages, lymphocytes and cytokines play a pivotal role in both development and progression of Th1-mediated autoimmune diseases, and a direct role in the destruction of thyroid follicles and follicular cell function in autoimmune thyroiditis. Integrins are integral membrane receptors involved in cell-extra-cellular matrix (ECM) interaction with both structural and signaling functions. The integrin-ECM interaction is necessary for the correct function and survival of thyroid follicular cells. The purpose of this study was to determine the effect of cytokine stimulation on integrin expression and signaling in the thyroid cell. Primary cultures from normal thyroids were treated with interferon-γ (IFN-γ), INF-α, tumor necrosis factor-α, interlukin 1a or these cytokines all together. Integrin expression, cell adhesion to fibronectin (FN) and FN-stimulated ERK phosphorylation were determined after cytokine treatment. IFN-γ and IFN-α were the most effective, reducing the expression of the integrin αvβ3 and slightly increasing the α3β1. Cell treatment with IFN-γ strongly impaired cell adhesion to FN. At the same time, the treatment with IFN-γ dramatically inhibited the stimulation of ERK phosphorylation induced by cell adhesion to FN. In conclusion, IFN-γ inhibits the expression of the integrin αvβ3, reducing the cell adhesion to FN and the following intracellular signaling in thyroid cells in culture. These results suggest that integrins may be a target of the infiltrating lymphocytes and have a role in the pathogenesis of autoimmune thyroiditis.

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INTRODUCTION

Hashimoto thyroiditis (HT) is a very common autoimmune thyroid disease, with a prevalence of 5% to 10% in the general population (1, 2). T-cell infiltration on histology and autoantibodies to thyroid peroxidase and thyroglobulin are the hallmark of HT (3, 4). This disease is initially characterized by goiter, followed by a gradual loss of thyroid function due to the progressive destruction of the normal follicular architecture of the gland and cell death. Thyroid infiltrating T lymphocytes have a crucial role in the pathogenesis of HT, by reacting with thyroid antigens and secreting inflammatory cytokines. In animal experimental models of autoimmune diseases, T helper cells type 1 (Th1) have been proposed to mediate tissue damage, mainly through intrathyroidal secretion of cytokines (4-6). The major cytokines produced by Th1 cells are interferon-γ (IFN-γ), IFN-α and interleukin 2, that are capable of inhibiting other interleukins produced by Th2 cells (7, 8). The analysis of intrathyroidal lymphocytes and in vitro experiments indicate that IFN-γ plays a crucial role in HT by acting on both immune cells and thyroid cells (4, 9, 10). Integrins, a family of cell surface receptors, are the most important mediators of cell adhesion to the extra-cellular matrix (ECM). The αβ integrin complex binds ECM by its extracellular domain and interacts with cytoskeletal proteins by its intracellular domain, contributing to the stability of the tissue structure (11). Some of these receptors colocalize with regulatory proteins in a subcellular structure called focal adhesion (12). Integrin activation by binding to ECM generates multiple intracellular signals that contribute to the regulation of many biological processes, including differentiation, growth and survival (13, 14). The integrin expression profile is cell type dependent and is modulated by a number of factors, including cell-to-cell contact, extracellular soluble factors and tumor transformation (15-17). Only few integrin types are expressed in normal thyroid cells (α3β1, αvβ3) (18, 19). Integrin activation by fibronectin (FN) has important biological effects in the thyroid cell, regulating the cytoskeletal organization and stimulating cell proliferation and survival (20, 21). The aim of this study was to investigate whether IFN-γ and other cytokines produced by thyroid infiltrating lymphocytes in HT, modulate the expression of FN binding integrins and their signaling in the thyroid cell. We found that INF-γ and INF-α reduce the expression the integrin αvβ3 and that IFN-γ strongly inhibits the signaling generated by FN binding.

MATERIALS AND METHODS

Tissues and cell cultures

Tissue specimens were obtained at surgery from the unaffected controlateral lobes of thyroid papillary carcinomas undergoing thyroidectomy. Patients were asked to give their consent to make their thyroid tissue available for experimental studies at the time of surgery. Tissues were chopped by scalpels in small pieces and digested by type IV collagenase (Sigma Chemical Co., St. Louis, MO; 1.25 mg/ml) in Ham's F-12

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medium and 0.5% BSA overnight at 4 C under rotation. Cells were pelleted by centrifugation at 150 x g for 5 min, washed twice in BSA-Ham's F-12 medium (BSA/F12), seeded in petri dishes, and cultured in 5% CO 2 atmosphere at 37 C in Ham's F-12 medium supplemented with 10% FCS. Medium was changed every 3– 4 days, and the cells to be examined were harvested by treatment with 0.5 m m EDTA in calcium- and magnesium-free PBS containing 0.05% trypsin (trypsin/PBS).

Antibodies and flow cytometric analysis

All the antibodies specific for single integrin subunits or heterodimers were mouse monoclonals purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein-conjugated antimouse IgG was purchased from Ortho (Raritan, NJ). Cells harvested from cell cultures by trypsin/PBS were incubated with monoclonal antibodies for 1 h at 4 C in PBS and 0.5% BSA (BSA/PBS), washed in the same buffer, and incubated again with the second fluorescein-conjugated antibody for 30 min at 4 C. Cells were resuspended in BSA/PBS and analyzed by flow cytometry. Single cell suspensions were analyzed by a FACScan (Becton Dickinson, Mountain View, CA). Forward scatter vs. side scatter analysis was performed on a logarithmic scale using a high forward threshold to cut off cellular and collagen debris.

Cell attachment assay

The assay was performed in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). The wells were filled with 100 ml of the appropriate dilution in PBS of FN (Collaborative Research, Bedford, MA). After overnight incubation at 4 C, the plates were washed with PBS, filled with 100 ml 1% heat-denatured BSA, and incubated for 1 h at room temperature. Then, plates were washed and filled again with 100 mL/well PBS, 0.9 m m CaCl 2, and 0.5 m m MgCl 2 containing 2 x 10^4 cells. After 30 min at 37 C, plates were gently washed three times with PBS, and the attached cells were fixed with 3% paraformaldehyde for 10 min followed by 2% methanol for 10 min and finally stained with 0.5% crystal violet in 20% methanol. After 10 min, plates were washed with tap water, the stain was eluted with a solution of 0.1 m sodium citrate, pH 4.2, in 50% ethanol, and the absorbance at 540 nm was measured by a spectrophotometer. Where indicated, the cells were coincubated with 500 μg/ml of integrin/FN binding inhibitory peptide RGSP (Gly-Arg-Gly-Asp-Ser-Pro) or the ineffective peptide RGE (Gly-Arg-Gly-Glu-Ser-Pro), Telios (San Diego, CA). All experiments were performed in quadruplicate.

Western blot

The cells were lysed in Laemmly buffer [125 mm Tris (pH 6.8), 5% glycerol, 2% SDS, 1% 2β-mercaptoethanol, and 0.006% bromophenol blue] and resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp., Bedford, MA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine, and after three washes, the membranes were incubated for 1 h at 4 C with mouse monoclonal primary antibodies in PBS. After three washes filters were incubated for 1 h at 4 C with a horseradish peroxidase-conjugated antimouse secondary antibody. After a final wash, protein bands were detected by an enhanced chemiluminescence

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system (ECL) (Amersham Pharmacia Biotech, Rainham, UK). Mouse monoclonal antibodies to ERK and phosphorylated ERK were from Santa Cruz Biotechnology, Inc.

RESULTS

Effect of cytokines on the expression of FN receptors of the integrin family in thyroid cells.

Normal thyroid follicular cells were cultured for a maximum of 14 days *in vitro* before the experiments. All experiments were performed on cells at 75% confluence to minimize cell-to-cell contact. The cells were treated for 3 days with IFN-γ, IFN-α, TNF-α and IL-1a, or with a mix of all the cytokines, then integrin expression was assessed by flow cytometry with specific antibodies for the subunits β1, α1, α2, α3, α4, α5, α6, and the heterodimers αvβ3, and α5β1 (Fig. 1). The expression of the α3 and β1 subunits were slightly increased by INF-γ and TNF-α, whereas the cooperative effect of all 4 cytokines was more robust. All cytokines were ineffective on the expression of the subunits α1, α2, α4, α5, α6 and on the heterodimer α5β1. More significant was the effect of INF-γ and INF-α on the αvβ3 heterodimer that was reduced (by 40% and 37% respectively). Also for this integrin the concurrent action of the 4 cytokines produced a more evident effect (56% reduction of expression in untreated cells).

Effect of cytokines on thyroid cell binding to FN.

The integrins α3β1 and αvβ3 are FN receptors and enable cell adhesion to immobilized FN (22). However, the affinity of integrin receptors for their ligands is subjected to the action of different factors (17). To investigate whether IFN-γ changed the ability of thyroid cells to attach to FN, thyroid cells were treated with the cytokine for 3 days and cell attachment assays were performed in 96-well flat bottom microtiter plates coated with different concentrations of FN (Fig. 2). Cell adhesion in the presence of the the RGDSP peptide, a specific inhibitor of integrin binding to FN, was minimal, demonstrating the integrin involvement. IFN-γ treated cells showed a drastic reduction of attachment to FN. The observation by inverted phase contrast microscopy showed a clear reduction of spreading of IFN-γ treated cells that displayed a more rounded shape, while untreated cells appeared more flat and polygonal. This data are consistent with the reduction of αvβ3 receptor induced by IFN-γ.

Effect of IFN-γ on FN-induced ERK phosphorylation.

FN/integrin binding to thyroid cells generates multiple cellular signals, including the the mitogen-activated kinase cascade leading to the activation of the extracellular-signal regulated kinase (ERK). To determine whether IFN-γ affects the integrin mediated ERK activation induced by FN, thyroid cells were untreated or treated with IFN-γ for 3 days, starved from serum overnight and plated onto immobilized BSA or FN. Cells treated with FCS were considered as positive control for ERK phosphorylation. After 30 minutes, the cells

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were lysed and phosphorylated ERK was evidenced by Western blot (Fig. 3). Adhesion to FN stimulated ERK phosphorylation with a magnitude comparable to FCS. The treatment with IFN-γ dramatically inhibited FN-stimulated ERK phosphorylation.

DISCUSSION

The decline of thyroid function and the destruction of thyroid follicular cells in HT is a slow progressive process only partly uncovered. Th1-mediated immune response plays a major role in this disease that culminates with fibrosis and loss of thyroid function (23). IFN-γ is a Th-1 cytokine produced intrathyroidally by infiltrating inflammatory cells, acting on thyroid follicular cells modulating their proliferation and the expression of different proteins including the major histocompatibility complex class II molecules, adhesion molecules and the sodium iodide symporter (7, 10, 24, 25). The relevant role of IFN-γ in the destruction of thyroid cells in HT is supported also by experimental evidences in animal models. Constitutive expression of IFN-γ in the thyroid transgenic mice resulted in severe hypothyroidism with reduced cell functionality and disruption of the thyroid architecture (26). Through its receptor, IFN-γ generates a complex intracellular signaling acting at transcription and translation level and ultimately modulates the cell behavior and the cell fate. The complex architecture of the thyroid gland, composed of cells organized in a follicular structure, is the result of the action of molecules involved in cell-to-cell and cell-ECM interactions. Integrins are the major receptors for ECM compounds and play a pivotal role in the stability of the tissue structure. Thyroid cells are polarized epithelial cells that require a continuous interaction with FN. Impaired adhesion to FN, besides the distortion of normal cell shape and tissue architecture, modifies intracellular signalings, inhibits cell proliferation and triggers a programmed cell death known as "anoikis" (19-22, 27, 28). In the present study, IFN-γ inhibited the cell membrane expression of the integrin αvβ3 the major FN receptor expressed by the thyroid cell. This integrin, when activated by binding to immobilized FN, activates multiple signalings including the MAPK cascade that leads to ERK activation and ultimately promotes cell proliferation and inhibits the anoikis. Although the expression of the α3β1 integrin was slightly increased by the treatment with IFN-γ, the decreased expression of αvβ3 prevailed, and the cell adhesion to immobilized FN resulted reduced. More importantly, the reduced FN/αvβ3 binding induced by IFN-γ determined a reduced signaling, as evidenced by the very weak stimulation of ERK phosphorylation in response to FN adhesion. Thyroid cell adhesion to FN promotes cell proliferation and survival through a common and then diverging signal pathway (19). The inhibition of this pathway, the same of many other growth and survival factors, makes the thyroid cell more sensitive to damaging agents. In conclusion, our study demonstrates that IFN-γ inhibits the expression of the integrin αvβ3, reducing the cell adhesion to FN and the following intracellular signaling in thyroid cells in culture.

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These results suggest that integrins may be a target of the infiltrating lymphocytes and have a role in the pathogenesis of autoimmune thyroiditis.

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