Molecular Pathways Involved in the Antineoplastic Effects of Calcitriol on Insulinoma Cells

FRANCESCALA GALBIATI, LUCA POLASTRI, BERNARD THORENS, PHILIPPE DUPRAZ, PAOLO FIORINA, UGO CAVALLARO, GERHARD CHRISTOFORI, AND ALBERTO M. DAVALLI

Division of General Medicine, Unit of Endocrinology and Metabolic Disease, San Raffaele Scientific Institute (F.G., L.P., P.F., A.M.D.), 20132 Milan, Italy; Institute of Pharmacology, University of Lausanne (B.T., P.D.), CH-1005 Lausanne, Switzerland; and Institute of Biochemistry and Genetics, University of Basel (U.C., G.C.), CH-4051 Basel, Switzerland

We have previously reported that in tumorigenic pancreatic β-cells, calcitriol exerts a potent antitumorigenic effect by inducing apoptosis, cell growth inhibition, and reduction of solid β-cell tumors. Here we have studied the molecular pathways involved in the antineoplastic activity of calcitriol on mouse insulinoma βTC3 cells, mouse insulinoma βTC expressing or not expressing the oncogene p53, and βTC-tet cells overexpressing or not the antiapoptotic gene Bcl2. Our results indicate that calcitriol-induced apoptosis was dependent on the function of p53 and was associated with a biphasic increase in protein levels of transcription factor nuclear factor-κB. Calcitriol decreased cell viability by about 40% in p53-retaining βTC and in βTC cells; in contrast, βTC p53−/− cells were only minimally affected. Calcitriol-induced cell death was regulated by members of the Bcl-2 family of apoptosis regulatory proteins, as shown by calcitriol-induced up-regulation of proapoptotic Bax and Bak and the lack of calcitriol-induced cytotoxicity in βTC-overexpressing insulinoma cells. Moreover, calcitriol-mediated arrest of βTC cells in the G1 phase of the cell cycle was associated with the abnormal expression of p21 and G/M-specific cyclin B2 genes and involved the DNA damage-inducible factor GADD45. Finally, in βTC3 cells, calcitriol modulated the expression of IGF-I and IGF-II genes. In conclusion, these findings contribute to the understanding of the antitumorigenic effects of calcitriol on tumorigenic pancreatic β-cells and further support the rationale of its utilization in the treatment of patients with malignant insulinomas. (Endocrinology 144: 1832–1841, 2003)

Today it is well recognized that the active form of vitamin D$_3$ (1,25-dihydroxyvitamin D$_3$ [1,25-(OH)$_2$D$_3$]), also known as calcitriol, acts as an effective regulator of cell growth and differentiation in a number of different cell types, including neoplastic cells (1). In the past decade several studies have demonstrated the antineoplastic effect of calcitriol on a variety of cancer cell lines, supporting its potential use in the treatment of diverse types of malignancies. Malignant insulinomas are rare endocrine tumors that, in contrast to benign β-cell adenomas, are rarely cured by surgery (2). In addition, malignant insulinomas respond poorly to conventional chemotherapy and treatment with somatostatin (or somatostatin analogs), which usually has been effective on other types of endocrine tumors (3, 4). Moreover, hypoglycemia associated with malignant insulinomas is generally unresponsive to agents that inhibit insulin release, such as diazoxide and certain calcium channel blockers (5–7).

We have recently reported that in murine insulinoma βTC3 cells, calcitriol induces growth inhibition and apoptosis (8). In the same study we have shown that the cytotoxic effects of calcitriol are limited to β-cells with a malignant phenotype (βTC3), whereas benign human insulinoma cells and normal human islets are unaffected. We have also demonstrated that a short course of treatment with calcitriol reduces significantly the mass of β-cell tumors in transgenic RIP1Tag2 mice (8), a well characterized model of β-cell tumorigenesis (9).

The nuclear vitamin D receptor (nVDR) is a member of the nuclear receptor superfamily that functions as a transcriptional factor and is also present in pancreatic β-cells (10). Calcitriol acts mainly through the so-called genomic pathway, which involves binding of the hormone to its nVDR. The complex calcitriol/nVDR binds to vitamin D-responsive elements in the promoter region of target genes and modulates their transcription. Vitamin D-responsive elements have also been identified in the promoter region of nonclassical vitamin D target genes such as calbindin D (11), p21 (12), c-Fos, and TGFβ2 (13). It has now become evident that calcitriol also induces nongenomic responses through the activation of a putative, yet unidentified, membrane receptor (14, 15). Indeed, calcitriol increases the intracellular levels or activities of several signaling molecules, including protein kinase C (PKC), Raf, MAPK, and Src kinases (16–18).

We recently reported that in βTC3 cells, calcitriol activates the MAPK cascade and induces PKC activation via a nongenomic pathway (8). In βTC3 cells, MAPK activation contributes to calcitriol-induced cytotoxicity, as MAPK kinase (MEK) inhibition with U0126 significantly prevents this effect (8). We also reported that calcitriol increases caspase-3 activity in βTC3 cells (8), but no particular efforts were posed in that study to explore in more detail the mechanisms responsible for the antineoplastic effect of calcitriol on these cells.

The aim of this study was to elucidate the molecular pathways involved in the antitumorigenic effects of calcitriol on βTC3 cells. The results show that calcitriol increased the levels of p53 protein and p53 phosphorylated at serine 15, an effect that was prevented by staurosporine, but not by the
MEK inhibitor (U0126). Moreover, calcitriol-induced βTC₃ cell apoptosis was associated to a biphasic increase in the protein levels of nuclear factor-κB (NFκB), which appears to be a prosurvival cell adaptation. Calcitriol transcriptionally activated several p53-regulated genes involved in the regulation of cell cycle (p21 and G₂/M-specific cyclin B2), DNA damage (GADD45), and apoptosis (Bak and Bax). Calcitriol-induced insulinoma cell death was significantly reduced in p53 null βTC (βTC p53⁻/⁻) cells and was completely prevented by Bcl-2 overexpression. Finally, exposure of βTC₃ cells to calcitriol altered the expression of the survival factors (IGF-I and IGF-II).

Materials and Methods

Cell lines and cultures

βTC₃ were originally provided by Shimon Efrat (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). βTC₃, βTCtet, and βTCtet/bcl2 were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 IU/ml streptomycin/penicillin. βTC p53⁻/⁻ and βTC p53⁻/⁺ were cultured in DMEM supplemented with 20% fetal calf serum, 2 mM glutamine, and 100 IU/ml streptomycin/penicillin. Cultures were performed under standard humidified conditions of 5% CO₂ and 95% air at 37°C.

Western blot analysis

βTC₃ cells were seeded at a density of 2 × 10⁵ onto 10-cm² tissue culture plates and allowed to attach and grow for 48 h. The medium was then replaced with fresh medium containing vehicle (ethanol; final concentration, 0.04%) or increasing calcitriol concentrations (10, 100, and 1000 nM). After 20 min and 4, 8, 24, and 48 h, cells were harvested and lysed in 200 µl lysis buffer (30 mM Tris-HCl, 5 mM EDTA, 250 mM sucrose, 1% Triton X-100, 1 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml aprotinin). After 1 h at 4°C, lysates were centrifuged at 13,000 rpm for 5 min, and the extracted proteins were analyzed by Western blotting using the following antibodies: anti-p53 (rabbit antibody; 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-panp3 (goat antirabbit; 1:100; Santa Cruz Biotechnology, Inc.), anti-phospho-p53 Ser 15 (rabbit polyclonal antibody; 1:100; Santa Cruz Biotechnology, Inc.), and antiaxon (goat antirabbit; 1:100; Sigma-Aldrich Corp., St. Louis, MO) commercial antibodies.

The levels of phosphorylated p53 protein were measured in βTC₃ cells after 20 min exposure to calcitriol (1000 nM) in the presence or absence of MEK inhibitor (U0126, 2 nM) and PKC inhibitor (staurosporine, 5 nM). Extracted proteins were analyzed by immunoblotting with antibodies against anti-phospho-p53 Ser 15 (rabbit polyclonal antibody; 1:100; CELBIO, New England Biolabs, Inc., Beverly, MA). Western blot bands were quantitatively analyzed by scanning acquisition (ScanJet 3500C, Hewlett-Packard Co., Palo Alto, CA) following the manufacturer’s instructions. The different patterns of gene expression in βTC₃ cultured for 48 h in the presence of increasing concentrations of calcitriol (10, 100, and 1000 nM). Total cellular RNA was prepared with the RNAfast RNA isolation system (M-Medical, Firenze, Italy), and 20 µg RNA from each sample were electrophoresed on 1% denaturing agarose gel. Blots were sequentially hybridized with a human IGF-I cDNA probe (a gift from Dr. Antonio Torsello, University of Milan, Milan, Italy), a rat IGF-II cDNA probe (provided by Dr. Steen Gameltoft, Bispebjerg Hospital, Copenhagen, Denmark), a human GADD45 cDNA probe (provided by Dr. Michael O’Reilly, University of Rochester, Rochester, NY), and a 18S cDNA probe. Relative expression levels of IGF-I, IGF-II, GADD45, and 18S were determined by densitometric analyses.

Statistical analysis

In vitro studies consisted of a minimum of three independent experiments, each carried out at least in duplicate. Datasets were expressed as the mean ± SE. Statistical analysis was performed using the unpaired t test for pairwise comparisons or one/two-way ANOVA (Tukey post hoc test), as appropriate. Statistical significance was considered at P < 0.05.

Results

Genes differentially expressed by βTC₃ cells cultured in the presence of calcitriol

Gene array analysis was performed to identify genes differentially expressed upon treatment of βTC₃ cells with calcitriol. Exposure to 1000 nM calcitriol induced the differential expression of a number of genes; some are known to be functional in the regulation of the cell cycle and apoptosis, and some are survival factors, all consistent with calcitriol’s antineoplastic effect on βTC₃ cells (Tables 1 and 2). Seven of the 588 genes tested were switched on/off by calcitriol.

<table>
<thead>
<tr>
<th>Table 1. Genes up- and down-regulated by 48 h of exposure to 1000 nM calcitriol in βTC₃ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold increase/ decrease</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± SE; n = 3 independent experiments; P < 0.05 for each gene listed in the table.
TABLE 2. Genes turned on and off by 48 h of exposure to 1000 nM calcitriol in βTC₃ cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Calcitriol effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADD45</td>
<td>On</td>
</tr>
<tr>
<td>SRY-box containing gene 4</td>
<td>On</td>
</tr>
<tr>
<td>Transcription factor UBF</td>
<td>Off</td>
</tr>
<tr>
<td>IRS-1</td>
<td>On</td>
</tr>
<tr>
<td>ACE</td>
<td>On</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Off</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>On</td>
</tr>
</tbody>
</table>

n = 3 independent experiments.

Calcitriol increases p53 and phosphorylated (Ser¹⁵) p53 protein levels in βTC₃

We have previously shown that the MAPK pathway is nongenomically activated by calcitriol in βTC₃ (8). Because of the well established link between p53 signaling and the MAPK cascade (21), we investigated whether the p53 pathway was involved in the antineoplastic effect of calcitriol on insulinoma cells. After 48 h of culture in the presence of increasing calcitriol concentrations, whole cell lysates were collected, and p53 levels were assessed by Western blot. As shown in Fig. 1A, p53 protein doubled after exposure to 100 nM calcitriol. Increasing the calcitriol concentration to 1000 mM did not further increase p53 protein levels. At 10 nM, calcitriol induced only a modest, nonsignificant increase in p53 protein.

Calcitriol also induced an increase in the levels of p53 phosphorylated at Ser¹⁵ to about 200% of control values by 20 min after treatment with 1000 mM calcitriol (Fig. 1B). This effect was prevented by the PKC inhibitor staurosporine, but not by the MEK inhibitor U0126 (Fig. 1B).

βTC₃ p53⁻/⁻ cells are less susceptible to calcitriol-induced cytotoxicity

To further investigate the involvement of p53 in calcitriol-induced insulinoma cell death, we used p53-null βTC cells (βTC p53⁻/⁻), which have been derived from tumors in RIP1Tag2/p53⁻/⁻ mice (22). Forty-eight hours of culture in 1000 nM calcitriol induced a significant decrease in the number of viable control βTC p53⁺/⁺ cells, quantitatively similar to what was observed in βTC₃ cells. In contrast, βTC p53⁻/⁻ cells were partially resistant to the cytotoxic effects of calcitriol. Increasing the calcitriol concentration to 1000 nM calcitriol induced a significant decrease in the number of viable control βTC p53⁺/⁺ cells, quantitatively similar to what was observed in βTC₃ cells. In contrast, βTC p53⁻/⁻ cells were partially resistant to the cytotoxic effects of calcitriol.
citriol, exhibiting only a modest decrease in the number of viable cells (Fig. 1C).

**Calcitriol increases p21 protein levels in βTC₃**

Antiproliferative signals, including serum deprivation or DNA damage, result in transcriptional activation of p21, a well known inhibitor of cyclin-dependent kinase (cdk2). p21, a bona fide transcriptional target gene of p53, was significantly up-regulated by calcitriol, as demonstrated by the gene array (Table 1) and Western blots (Fig. 2A). Calcitriol increased p21 protein in βTC₃ cells in a dose-dependent manner to 160%, 180%, and 210% of control values after culture in 10, 100, and 1000 nm calcitriol, respectively (Fig. 2A).

**Calcitriol increases NFκB protein levels**

It has been previously shown that induction of p53 causes an activation of NFκB that correlates with the ability of p53 to induce apoptosis (23). Therefore, we determined the protein levels of NFκB in βTC₃ cells after 48 h of culture in the presence of increasing concentrations of calcitriol. NFκB protein levels increased to 200% of control values at 10 nm calcitriol, a level that did not significantly change with higher concentrations of calcitriol (Fig. 2B).

**Time course of the expression levels of p53, NFκB, IκB, and p21 protein upon calcitriol exposure**

The protein levels of p53, NFκB, IκB and p21 were measured 20 min and 4, 8, 24, and 48 h after exposure to 100 nm calcitriol and were compared with control levels. As shown in Fig. 3A, the increase in p53 protein levels was significant after 8 h and then remained stable for the entire period of stimulation. Conversely, NFκB protein levels showed a biphasic pattern of activation with two peaks, at 8 and 48 h, that were separated by a decrease to control levels at 24 h. A similar biphasic pattern of NFκB activation was recently reported (24). Interestingly, IκB protein levels showed the opposite pattern, with a decrease at 8 h (~70% of control) and a peak at 24 h (~200% of control; Fig. 3C). Finally, p21 showed a significant increase in protein levels only after 48 h, suggesting that the transcriptional regulation exerted by calcitriol on this gene is exclusively mediated by its classical genomic effect (Fig. 3D).

**NFκB inhibition with PDTC fails to protect βTC₃ cells from calcitriol-induced apoptosis**

To test whether NFκB activation was involved in calcitriol-induced apoptosis, treatment with calcitriol was performed in the presence or absence of PDTC (25, 26), an inhibitor of NFκB DNA-binding activity (Fig. 4A). PDTC alone was toxic for βTC₃ cells at the lowest concentration known to have a biological effect (20 μM). Moreover, when calcitriol and PDTC were added together, the number of viable cells decreased further, indicating an additive effect of calcitriol and PDTC on βTC₃ cells.

**Calcitriol does not increase NFκB protein levels in βTC p53⁻/⁻ cells**

To better understand the relationship between p53 and NFκB, we measured NFκB protein levels in βTC p53⁻/⁻ and p53⁻/⁻ cells, which, as previously shown, are partially resistant to calcitriol-induced apoptosis. As shown in Fig. 4B, 8 and 48 h after calcitriol exposure, NFκB protein levels were increased in βTC₃ and βTC p53⁻/⁻ cells, but not in p53⁻/⁻ cells.

**Calcitriol increases GADD45 gene expression**

DNA damage and environmental stress activate GADD45, a p53-regulated gene that is thought to play a role in growth arrest and cell death. Gene array analysis showed that calcitriol switched on GADD45 expression in βTC₃ cells (Table 1).
2). Even though a low level of expression of GADD 45 was detectable by Northern blot analysis, the inducible effect of calcitriol on GADD45 expression (Fig. 5) was confirmed. After 48 h of treatment, GADD45 mRNA increased to 206%, 236%, and 217% in the presence of 10, 100, and 1000 nM calcitriol.

Calcitriol up-regulates Bak and Bax gene expression in βTC3 cells, whereas Bcl-2 overexpression completely prevents calcitriol-induced cytotoxicity

Gene array analysis revealed an increased expression of the proapoptotic genes Bak and Bax (Table 1). We performed relative quantitative multiplex RT-PCR to confirm these results. We used two primer sets in a single PCR reaction: one set to amplify the cDNA of interest (Bak and Bax) and a second to amplify an invariant endogenous control (18S). These experiments revealed that after 48 h of culture in the presence of increasing calcitriol concentrations Bak and Bax expression were significantly increased in βTC3 cells, with a maximal effect achieved at the lowest (10 nM) calcitriol concentration (Fig. 6, A and B).

The βTC3 insulinoma cell line (27) was also sensitive to calcitriol-induced cytotoxicity; however, forced expression of Bcl-2 in βTC3/Bcl-2 cells (28) completely abolished this effect (Fig. 6C). These results suggest that calcitriol-induced apoptosis of tumor β-cells is modulated by members of the Bcl-2 family of genes.

Calcitriol induces IGF-I, but down-regulates IGF-II, gene expression

It has been shown that the IGF-I/IGF-II signaling pathway contributes to the antineoplastic effect of vitamin D in a variety of cancer cell lines (29–31). Therefore, we studied the expression of IGF-I and IGF-II by RT-PCR and Northern blotting in βTC3 cells cultured for 48 h in the presence of increasing calcitriol concentrations. In βTC3 cells, calcitriol induced the expression of IGF-I mRNA, which was completely silenced in control condition (Fig. 7, A and B), an effect that was already evident at 10 nM. In contrast, calcitriol modestly, yet significantly, down-regulated the expression of two IGF-II mRNA isoforms (Fig. 7D), thereby confirming the data obtained by RT-PCR (Fig. 7, A and C).

Discussion

Besides its central role in the maintenance of calcium homeostasis and bone mineralization, calcitriol is an important modulator of cell growth and differentiation in a variety of cell types. We previously described a wide spectrum of negative effects exerted by calcitriol on mouse insulinoma βTC3 cells, ranging from the induction of growth arrest and apoptosis of cultured tumor β-cell lines in vitro to the reduction of β-cell tumors in vivo in RIP1Tag2 transgenic mice (8).

To learn more about the mechanisms responsible for the antineoplastic effects of calcitriol on insulinoma cells, we set out to identify genes that are differentially expressed by βTC3 cells upon treatment with calcitriol. Calcitriol induced the differential expression of a number of genes known to be important in the regulation of the cell cycle. βTC3 cells exposed to calcitriol exhibited a 50% decrease in the expression of the cyclin B2 gene, which is a central regulator of the progression from the G2 phase to mitosis (32). Another cell cycle gene differentially expressed by βTC3 exposed to cal-
citriol was p21, whose mRNA expression was modestly, yet significantly, increased by calcitriol. Calcitriol-mediated expression of p21 was confirmed by Western blotting, revealing a dose-dependent increase in p21 protein in response to increasing calcitriol concentrations. Interestingly, time-course experiments showed that p21 protein levels increased 24 h after calcitriol exposure, suggesting that this particular effect of calcitriol is mediated by the classic genomic pathway. p21 is an inhibitor of cdk and inhibits the formation of cyclin-cdk complexes necessary for transition from the G1 to the S phase of the cell cycle (33). Together, these findings are consistent with the previously reported growth inhibitory effect of calcitriol on HT29 cells, characterized by an increased number of HT29 cells found in the G0/G1 fraction and a contemporary decrease in those in S and G2/M phases (8).

p53 is the most important tumor suppressor protein identified to date and can influence the cell cycle in several ways. It can cause growth arrest and apoptosis by forming complexes with other proteins, and it also acts as a transcription factor (34–36). Noteworthy, both cyclin B2 and p21 are transcriptional targets of p53 (33, 34). Simian virus 40 large T antigen, a potent oncoprotein present in HT29 and HT10 cells, exerts its oncogenic effect in part through binding to and inactivating the tumor suppressor gene products p53 and retinoblastoma (37). In HT29, calcitriol induced a 2-fold increase in p53 protein levels, an effect that was already maximal at 100 nM. Time-course experiments showed that at this concentration of calcitriol the increase in p53 protein levels was already evident after 8 h of stimulation, even though the maximal effect occurred at 48 h. It has been shown that in response to DNA damage, p53 protein levels and activity increase mainly as a result of its phosphorylation (35), which can be induced by DNA damage-sensing kinases as well as by MAPKs (39, 40). Therefore, we explored whether calcitriol...
would induce p53 phosphorylation in insulinoma βTC3 cells. We decided to study phosphorylation at serine 15, because this site of phosphorylation prevents the interaction of p53 with Mdm2, a protein that can down-regulate p53 via ubiquitin-mediated proteolysis. After 20 min of calcitriol treatment, βTC3 cells showed a 2-fold increase in phospho-p53 protein levels. This effect was prevented by staurosporine, but not by UO126, suggesting that MAPKs are not involved in this specific phosphorylation, whereas PKC may be an upstream activator of p53 kinases. Taken together, these data suggest that calcitriol, by increasing the p53 level and activity by combining nongenomic and genomic effects, may reduce the oncogenic potential of T antigen in βTC3 and βTC insulinoma cells.

Previous reports indicated that βTC cell lines derived from tumors of RIP1Tag2/p53-null mice have an apoptotic incidence comparable with normal βTC lines and suggested that p53-independent apoptotic pathways are used in βTC cells (41, 22). To learn more about the role of p53 in calcitriol-induced insulinoma cell death, we performed a set of experiments with βTC p53-null cells (βTC p53−/−). In βTC p53−/− cells, calcitriol induced only a modest decrease in the number of viable cells (~10% reduction), significantly lower than that observed in control βTC p53+/+ cells (~40% reduction). Therefore, in contrast to the majority of classic chemotherapeutic agents (22), calcitriol needs a functional p53 to fully exert its antineoplastic activity in insulinoma cells. Similar results were observed in glioma cells (42), but not in breast and colon cancer cells, where the antineoplastic

![GADD45 and 18S expression](image)

**Fig. 5.** Effects of calcitriol exposure on GADD45 gene expression. In βTC3 cells, 48 h of exposure to calcitriol significantly increased the mRNA levels of DNA damage-inducible GADD45 gene. The calcitriol effect was maximal at a concentration of 10 nM (#, P < 0.01 vs. control, by one-way ANOVA, post hoc Tukey test; mean ± SE; n = 3 experiments, each performed in triplicate).
effects of calcitriol and its analogs do not require the function of p53 (43, 44).

The role of NFκB in programmed cell death and cell cycle is still controversial. Reportedly, the proapoptotic or antiapoptotic effect of NFκB depends on the different cell types and the external stimuli applied (45). In the resting cell, NFκB is sequestered in the cytoplasm linked to its inhibitor protein, IκB. Upon activation, free NFκB increases as a result of IκB phosphorylation and degradation. Free NFκB translocates to the nucleus, binds its consensus sequences, and regulates the transcription of a variety of genes. We show here that calcitriol induces in ßTC3 cells a biphasic increase in NFκB protein, similar to what occurs in skeletal muscle cells (24). The first peak was observed after 8 h of stimulation and was associated with a significant decrease in IκB protein levels, which, as previously reported (45), might be consequent to IκB phosphorylation by MAPKs. Perhaps a similar series of events may occur in ßTC3 cells, as calcitriol can induce activation of the MAPK pathway (8). A second increase in NFκB protein levels was detected at 48 h and was associated with a decrease in IκB.

It has been previously reported that induction of p53 causes an activation of NFκB, which correlates with the ability of p53 to induce apoptosis (23). To exclude such possibility, we studied the pattern of NFκB activation in ßTC3 cells and the effect of NFκB inhibition with PDTC. As shown in Fig. 4B, calcitriol-induced increase in NFκB was completely absent in ßTC p53−/− cells at both 8 and 48 h. Moreover, as shown in other cell lines (46, 47), inhibition of NFκB decreased ßTC3 cell viability and amplified calcitriol-induced cytotoxicity. Taken together these data suggest that NFκB up-regulation is an antiapoptotic reaction mounted by these cells against calcitriol-induced cell death.

As previously reported in glioma cells (42), calcitriol up-regulated the expression of GADD45, another important p53-dependent regulator of cell fate (48), in ßTC3 cells. Calcitriol also increased the expression of Bak and Bax, two proapoptotic members of the Bcl-2 gene family (49).
This effect is consistent with the reported capability of p53 to transcriptionally activate Bax (50). The involvement of proapoptotic Bak and Bax may contribute to calcitriol-induced $\beta$TC$_3$ cell death by causing mitochondrial membrane damage. Overexpression of the antiapoptotic Bcl-2 gene completely prevented calcitriol-induced apoptosis. Hence, although multiple pathways may be involved in calcitriol-induced $\beta$TC$_3$ cell apoptosis, they can be modulated by the function of Bcl-2, which is known to play a pivotal role in the regulation of cell death by acting at the mitochondrial and pre- and postmitochondrial levels (51). It is noteworthy that Bcl-2 overexpression in breast cancer cells conferred complete protection against apoptosis induced by vitamin D compounds, which in these cells is a p53-independent event (43).

IGF-I and IGF-II are the most abundant growth factors in the body and act as potent survival factors. Exposure of $\beta$TC$_3$ to calcitriol induced the expression of IGF-I mRNA, that is undetectable in control cells, as shown by RT-PCR and Northern blotting. The induction of IGF-I expression was associated with a modest, but significant, down-regulation of the IGF-II gene in both its isoforms. Calcitriol-mediated IGF-II down-regulation is consistent with its antineoplastic effects. Similar results have been reported in prostate cancer cells, where vitamin D-mediated growth inhibition was associated with increased levels of IGF-binding protein-3 mRNA (52, 53). Calcitriol-induced IGF-II down-regulation may be particularly relevant in vivo in the prevention of insulinomas in transgenic RIP1Tag2 mice. In these mice, which represent a well-characterized model of multistage $\beta$-cell tumorigenesis, all the islets of Langerhans express simian virus 40 T antigen, yet only half of them become hyperplastic, and only a minority of these eventually progress to solid $\beta$-cell tumors (9, 54). Notably, in these mice the onset of proliferation strictly correlates with the expression of IGF-II in the $\beta$-cells (55). Perhaps, a precocious treatment of RIP1Tag2 mice with calcitriol may prevent this proliferative switch and thus the development of $\beta$-cell tumors.

Calcitriol-induced IGF-I expression is less compatible with the antineoplastic effect of calcitriol on $\beta$TC$_3$ cells. Nevertheless, calcitriol-induced IGF-I up-regulation in pancreatic $\beta$-cells is particularly appealing and may be relevant to the reported beneficial effect of calcitriol on the pathogenesis of type 1 diabetes. It has been shown that treatment with calcitriol prevents diabetes in the NOD mouse (56) as well as the recurrence of autoimmune destruction of syngeneic islet grafts (57). These effects have been related to the known immunomodulatory properties of vitamin D$_3$ on dendritic cells (58). However, our observation that calcitriol induces IGF-I up-regulation in tumor $\beta$-cells and the recent finding that $\beta$-cell-targeted IGF-I overexpression counteracts insulin-associated type 1 diabetes (59) suggest that calcitriol might exert its antidiabetic effect also by decreasing $\beta$-cell susceptibility to proinflammatory cytokines via IGF-I up-regulation.

In conclusion, calcitriol exerts a profound antineoplastic effect on mouse insulinoma cells, which is mediated by the abnormal expression of a series of genes involved in the control of cell cycle and cell death. The antineoplastic effect of calcitriol on insulinoma cells appears to depend mainly on the p53 pathway and to involve both the genomic and nongenomic pathways. These data support the rationale for testing calcitriol or its lower calcemic analogs in the treatment of patients with malignant insulinomas and possibly other p53 function-retaining endocrine tumors. Moreover, calcitriol-induced IGF-I overexpression, if confirmed to occur also in normal $\beta$-cells, may provide an additional rationale for using vitamin D compounds in the prevention of autoimmune diabetes and to promote the engraftment and survival of pancreatic islet allografts.

Acknowledgments

We thank Dr. Antonio Torsello (Department of Pharmacology, University of Milan, Milan, Italy) for the human IGF-I cDNA probe, Dr. Steen Gammeltoft (Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark) for the rat IGF-II cDNA probe, and Dr. Michael O’Reilly (University of Rochester, Rochester, NY) for the CADD45 cDNA probe.

Received September 30, 2002. Accepted January 28, 2003.

Address all correspondence and requests for reprints to: Dr. Alberto M. Davalli, Division of General Medicine, Unit of Endocrinology and Metabolic Disease, San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milan, Italy. E-mail: alberto.davalli@srs.it.

This work was supported by a grant from Ministry of Health of Italy (Projects RP98.52 and RP98.50; to F.G. and L.P.).

References


53. Mathiasen IS, Lademann U, Jaatela M 1999 Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res 59:4848–4856


Downloaded from endo.endojournals.org at Indonesia:Endo Jnls Sponsored on May 29, 2007