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Potentiation by vitamin D analogs of TNFα and ceramide-induced apoptosis in MCF-7 cells is associated with activation of cytosolic phospholipase A_2

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Abstract

Synthetic analogs of vitamin D induce apoptosis in cultured breast cancer cells and cause regression of experimentally-induced rat mammary tumors. To further elucidate the mechanisms involved, we have examined interactions between two vitamin D analogs (CB1093 and EB1089) and known mediators of apoptosis, TNFα and ceramide. Pretreatment of MCF-7 breast cancer cells with CB1093 and EB1089 substantially potentiated cytotoxic effects of TNFα as assessed by cell viability assay, DNA fragmentation and videomicroscopy. No significant changes in the levels of TNFα or TNF-RI transcripts were detected. CB1093 primed cells demonstrated enhanced responsiveness to cell permeable C_2-ceramide in terms of increased DNA fragmentation and loss of cell viability. Activation of cytosolic phospholipase A_2 ($cPLA_2$) has been implicated in TNFα-mediated apoptosis. As assessed by [^{3}H]-arachidonic acid release, cells primed for 48 h with CB1093 (50 nM) showed enhanced $cPLA_2$ activation in response to TNFα or ceramide. CB1093 treatment alone led to $cPLA_2$ activation and loss of cell viability which was inhibited by the specific inhibitor $AACOCF_3$. These results suggest that TNFα and vitamin D analogs share a common pathway leading to apoptosis involving $cPLA_2$ activation and/or ceramide generation.

Keywords: vitamin D analogs; breast cancer; $cPLA_2$; ceramide; apoptosis; TNFα

Abbreviations: CB1093, 20-epi-22(S)-ethoxy-23yne-24α,26α,27α-trihomo-1α,25-dihydroxyvitamin D_3; EB1089, 22,24-diene-24α,26α,27α-trihomo-1α,25-dixydroxyvitamin D_3; TNFα, tumor necrosis factor alpha; $cPLA_2$, cytosolic phospholipase A_2; AA, arachidonic acid

Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has been shown to possess many properties unrelated to its classical functions in the control of bone and mineral metabolism. Furthermore, $1,25(OH)_2D_3$ has been shown to inhibit growth and promote differentiation in a variety of normal and malignant cell types.[1–4] The observation that a high proportion of breast tumor biopsy specimens contain receptors for $1,25(OH)_2D_3$ and that the growth of cultured human breast cancer cells is inhibited in the presence of this vitamin D metabolite led to the suggestion that vitamin D derivatives might have potential as therapeutic agents in this and other malignancies. The use of conventional vitamin D metabolites in this setting is limited by side effects such as hypercalcemia, hypercalciuria and soft tissue calcification. Synthetic vitamin D analogs have been developed which retain the ability to control cell proliferation and differentiation but display reduced calcemic activity relative to $1,25(OH)_2D_3$ *in vivo*.[5,6] In addition, a number of these compounds have been tested for their effectiveness in inhibiting the growth of breast cancer.[7,8] We have carried out preclinical trials with a range of structurally modified compounds to assess effects on tumor growth *in vivo* using the rat model of hormone dependent breast cancer in which mammary tumors are induced with the carcinogen nitrosomethylurea. These studies demonstrated that a number of analogs including EB1089 and CB1093 cause regression of these experimental tumors.[9,10]

Tumor regression occurs when the rate of cell death is greater than the rate of cell proliferation and more recent studies have shown that induction of apoptosis (programmed or active cell death) may be a feature of the anti-tumor effects of certain vitamin D analogs. Several groups have presented evidence for the presence of DNA fragmentation, a key feature of apoptosis, in breast cancer cells treated with vitamin D derivatives.[11–14] Mechanisms by which vitamin D analogs promote apoptosis could involve suppression of cell survival signals and/or induction of genes that stimulate apoptosis. In the present study we have examined whether vitamin D analogs may potentiate responsiveness of breast cancer cells to known mediators of apoptosis. TNFα is a potent cytokine which demonstrates anti-tumor activity both *in vivo* and *in vitro* and induces apoptosis in a variety of tumor cell lines. Of the two TNF receptors, TNF-RI (p55) and TNF-RII (p75), the former is thought to promote cytotoxic effects. The binding of TNFα to its receptors is necessary but not sufficient for its cytotoxic action.[15,16] The signal transduction pathways involved in TNFα-mediated cell

death are obscure but these receptors are assumed to transduce signals across the plasma membrane. Studies have showed that the TNF-RI death domain specifically interacts with the intracellular adaptor protein TRADD, which in turn associates with another adaptor protein FADD/MORT1 and the newly formed complex can interact and activate caspase-8/FLICE leading to induction of apoptosis.[17,18]

The sphingomyelin pathway mediates signaling for apoptosis induced by several agents including TNFα.[19,20] Activation of sphingomyelinases hydrolyses sphingomyelin to generate ceramide which acts as a second messenger in distinct signaling pathways such as cell proliferation and apoptosis.[20–22] Cytosolic phospholipase A_2 (cPLA$_2$ or PLA$_2$-IV isoform) is involved in arachidonic acid (AA) release from its cellular storage in the phospholipid pool and plays an important role in cellular signaling (cellular proliferation and inflammation).[23,24] As with ceramide, it has been documented that activation of

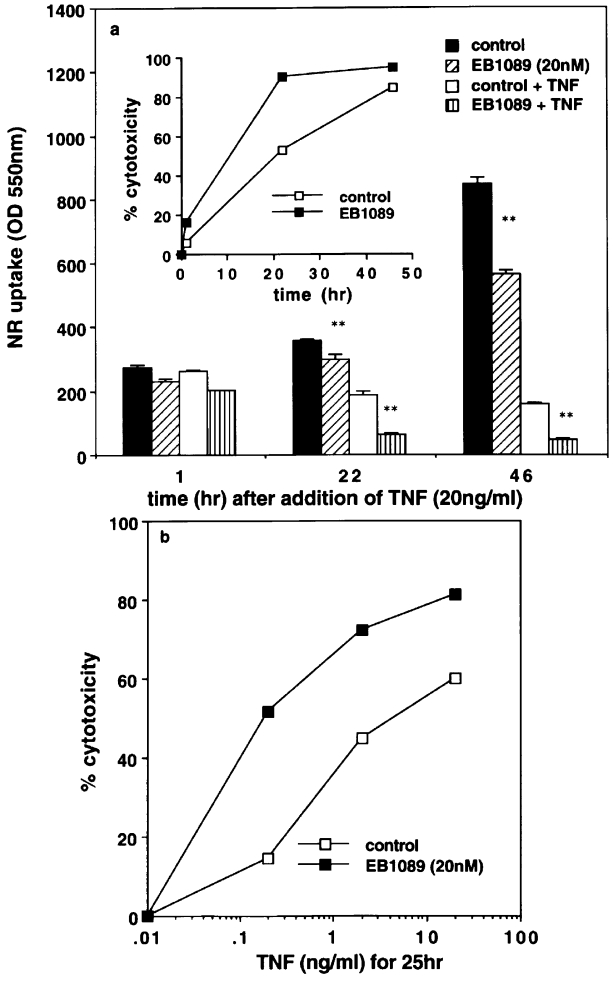


Figure 1 (**a**) Effects of pretreatment with vitamin D analog EB1089 on TNFα-induced cytotoxicity in MCF-7 cells. Cells were plated in 24 well plates and treated for 67 h with 20 nM EB1089 or ethanol vehicle followed by post-treatment with 20 ng/ml TNFα for up to 46 h. Results are expressed as mean ± S.E.M. of six replicate cultures. Percentage cytotoxicity was determined by neutral red dye assay as described in Materials and Methods. The difference between cultures treated with TNFα alone and TNFα plus EB1089 was significant at 22 and 46 h (**$P<0.001$). Differences between control and EB1089 treated cultures were significant at 22 and 46 h (**$P<0.001$). (**b**) Effects of increasing concentrations of TNFα on MCF-7 cell viability in cultures pretreated with EB1089. Cells were plated in 24 well plates and treated for 67 h with 20 nM EB1089 or ethanol vehicle followed by post-treatment with increasing concentrations of TNFα for 25 h

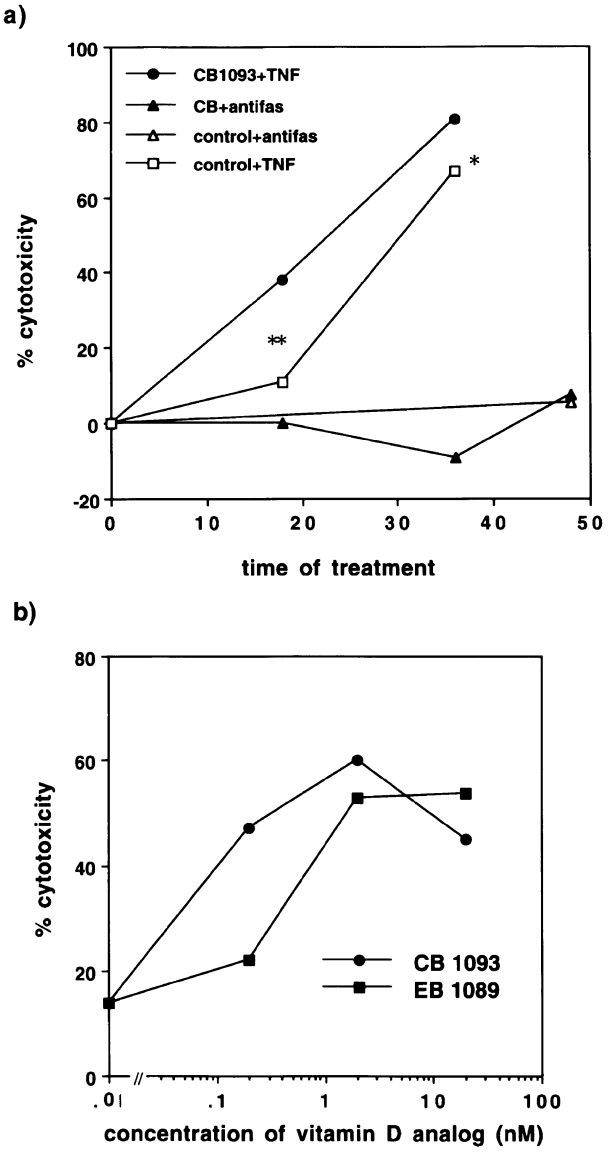


Figure 2 (**a**) Effect of pretreatment with vitamin D analogs on TNFα-induced cytotoxicity in MCF-7 cells. Cells were primed for 24 h with 20 nM CB1093 and then post treated with TNFα (5 ng/ml) or anti-Fas (250 ng/ml) for up to 48 h. Differences between cultures treated with TNFα alone and CB1093 plus TNFα were significant at 18 and 36 h (**$P<0.001$ and *$P<0.01$ respectively). Results are representative of three separate experiments. (**b**) Dose dependent effect of vitamin D analogs on TNFα-induced cytotoxicity. Cells were plated into 24 well plates and treated with increasing concentrations of EB1089 or CB1093 for 65 h followed by post-treatment with 10 ng/ml TNFα. Percentage cytotoxicity was determined by neutral red dye assay as described in Materials and Methods with six replicate cultures per treatment group. Results are representative of at least four separate experiments

$cPLA_2$ and release of AA as a second messenger is implicated in distinct signaling of TNFα-mediated apoptosis in several cell lines including MCF-7 cells. $cPLA_2$ is normally located in the cytoplasm and it translocates to the cell membrane in a calcium dependent or independent manner.[25,26] In order to define the cellular and molecular mechanisms involved in vitamin D-induced apoptosis in breast cancer cells, we have assessed the effects of two vitamin D analogs EB1089 and CB1093 on TNFα-induced apoptosis in MCF-7 cells. We now report that these vitamin D analogs potentiate responsiveness of breast cancer cells to TNFα and suggest that ceramide and/or $cPLA_2$ might be involved in a common pathway in TNFα and vitamin D mediated apoptosis.

Results

Vitamin D analogs potentiate the cytotoxic effects of TNFα

The cytotoxic effect of TNFα on MCF-7 cells and its modulation by synthetic vitamin D analogs is shown in Figures 1 and 2. MCF-7 cells were found to be responsive to induction of apoptosis by TNFα and this cytotoxic effect was augmented in cultures pretreated with the vitamin D analogs EB1089 and CB1093. Initially, the time course of TNFα-induced cytotoxicity in the presence or absence of the vitamin D analogs EB1089 and CB1093 was determined. As shown in Figure 1a, MCF-7 cell cultures preincubated with 20 nM EB1089 for 67 h and subsequently treated with 20 ng/ml TNFα achieved 90% cytotoxicity 22 h after addition of the cytokine, compared to

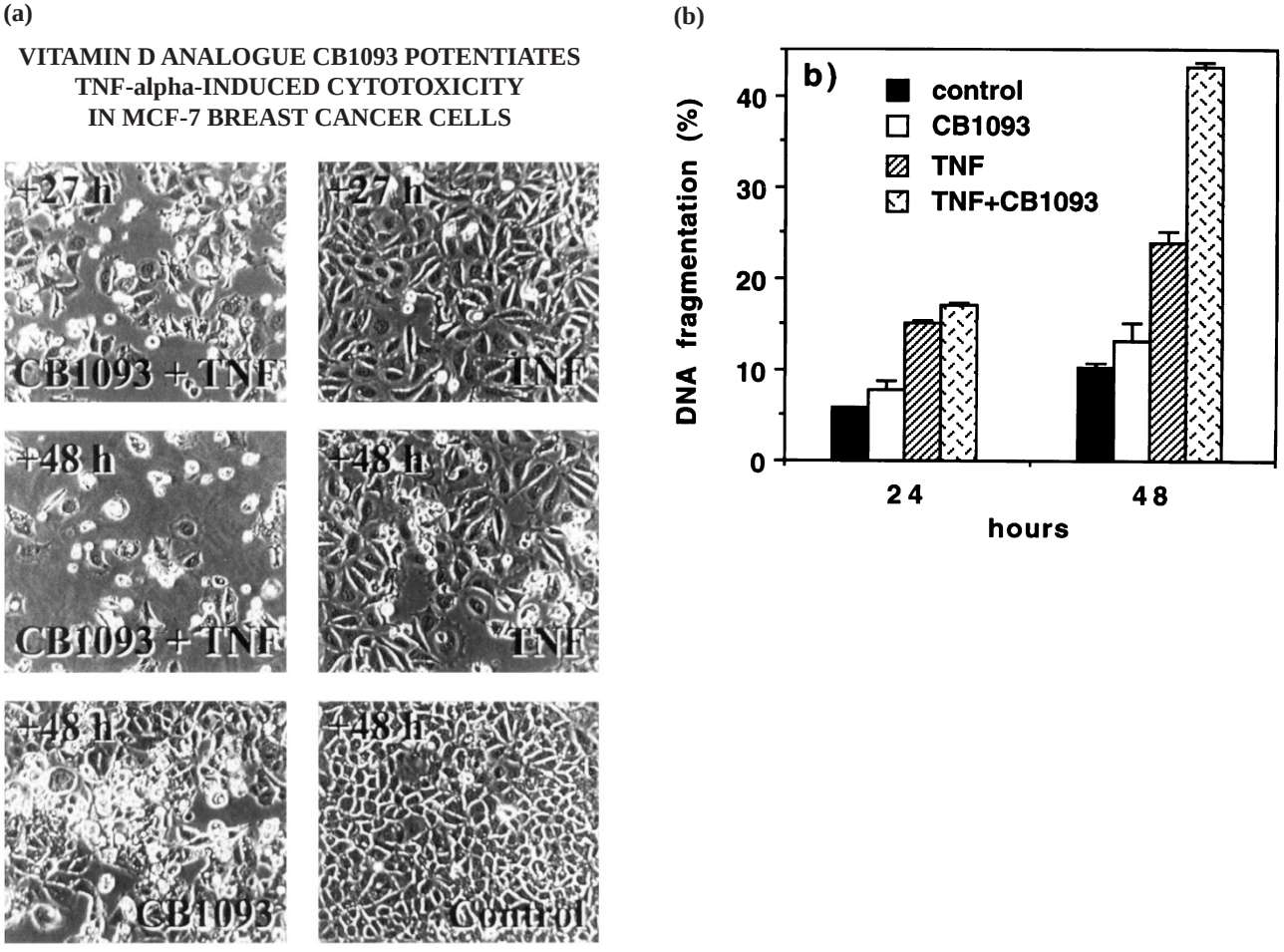


Figure 3 Videomicroscopy of apoptosis and DNA fragmentation in MCF-7 cells treated with CB1093 and TNFα. (**a**) MCF-7 cells were seeded in T25 tissue culture flasks and treated for 42 h with 25 nM CB1093 or ethanol vehicle. TNFα (10 ng/ml) was then added and cultures were incubated for a further 48 h. Typical fields from CB1093/TNFα (left top and middle panels) and control/TNFα treated cultures (right top and middle panels) were photographed over this period at 1/150 normal speed with a time-lapse videorecorder. At 48 h typical fields from control (right lower panel) and cultures treated with CB1093 alone (left lower panel) were similarly photographed. (**b**) MCF-7 cells were labeled with [^{3}H-*methyl*]-thymidine (0.5 μCi/ml) for 24 h, washed twice and then pretreated with CB1093 and finally exposed to TNFα for 24 and 48 h and assessed for DNA fragmentation as described in Materials and Methods. Results are the mean ± S.D. of three replicate estimations and are representative of three separate experiments

53% cytotoxicity seen with cultures preincubated in the absence of the vitamin D analog. In addition to reducing the time required to achieve substantial cytotoxicity, pretreatment with the vitamin D analogs led to a reduction in concentration of the cytokine required to induce significant cell death as illustrated in Figure 1b. Pretreatment of cells with 20 nM EB1089 for 67 h reduced the concentration of TNFα required for 50% cytotoxicity from 4 – 0.2 ng/ml. Similar potentiation of TNFα-induced cytotoxicity was seen in cultures pretreated with 20 nM CB1093 for 24 h and post-treated with 10 ng/ml TNFα for up to 36 h. However, no substantial cytotoxicity was seen with control or CB1093-primed cells exposed to 250 ng/ml anti-Fas for up to 48 h (Figure 2a). Regarding the relative potency of the two vitamin D analogs in potentiating TNFα-induced apoptosis, Figure 2b demonstrates that CB1093 is more effective than EB1089 in promoting responsiveness to the cytokine. Following post-treatment with 10 ng/ml TNFα for 23 h, 50% cytotoxicity was observed in cells pretreated with 0.3 nM CB1093 compared to 1.6 nM EB1089. This order of potency is similar to that which we have observed with CB1093 and EB1089 in inducing active cell death in MCF-7 cells after 5 days of treatment with the vitamin D analogs alone.[10] Videomicroscopy confirmed that the accelerated loss of cell viability in cultures pretreated with vitamin D analogs was due to apoptosis. Following 27 h exposure of CB1093-primed cultures to TNFα, morphological features of active cell death were observed including detachment and cell shrinkage (Figure 3a). Furthermore data obtained from DNA fragmentation analysis correlated well with videomicroscopy. CB1093-primed MCF-7 cells demonstrated enhanced responsiveness to TNFα in terms of increased levels of TNFα-induced DNA fragmentation at 24 or 48 h respectively (Figure 3b). We next assessed the minimum time of pretreatment with the vitamin D analogs which is required to observe potentiation of TNFα cytotoxicity. Addition of 20 nM EB1089 or CB1093 2 h prior to TNFα treatment yielded similar potentiation to that seen with 24 h pretreatment (data are not shown). No significant enhancement of TNFα effects was seen when the analogs were added 17.5 h after the cytokine.

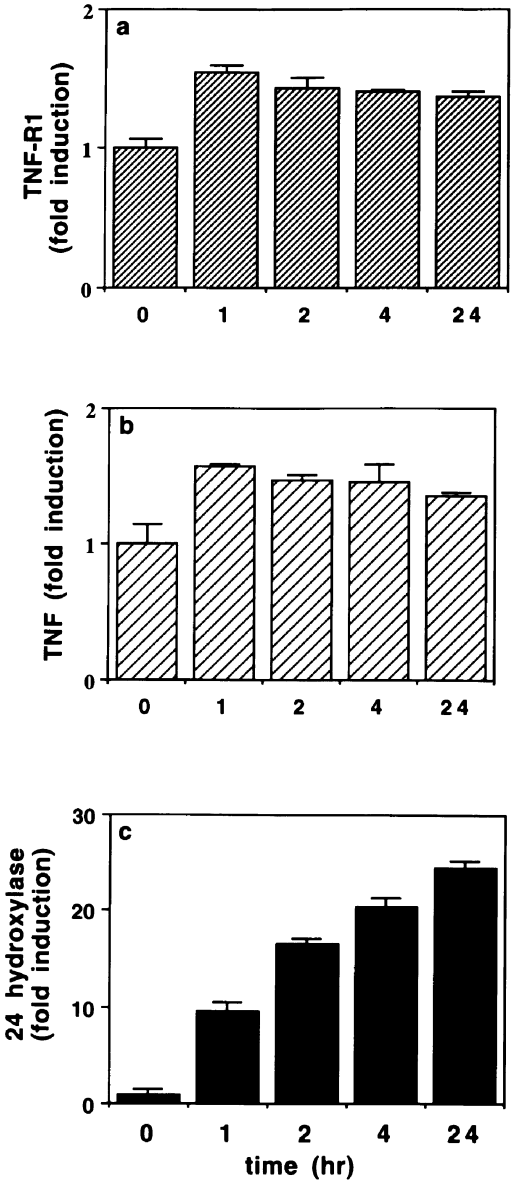


Figure 4 TNFα, TNFR-I and 24-hydroxylase mRNA expression in MCF-7 cells. Cells were treated with CB1093 for 1, 2, 4 and 24 h and harvested. Levels of expressed TNF-RI (**a**), TNFα (**b**) and 24-hydroxylase mRNA (**c**) were obtained by semi-quantitative RT – PCR in relation to β2 microglobulin mRNA expression. Data shown are based on three separate RNA preparations per treatment condition and PCR reactions were performed in duplicate

Effects of CB1093 on TNF-RI and endogenous TNFα expression

To determine if the mechanism by which vitamin D analogs enhance responsiveness to the cytokine occurred by promoting recognition of the TNFα signal, effects on expression of TNF-RI were examined at the mRNA level. MCF-7 cell cultures were treated with ethanol vehicle or CB1093 (100 nM) for 1 – 24 h and level of induction of TNF-RI transcripts determined by RT – PCR. Only a modest (1.5 fold) increase in the induction of TNF-RI transcripts was observed at 1 h in comparison to a tenfold induction of 25-hydroxyvitamin D_3-24-hydroxylase, a gene previously demonstrated to be induced by $1,25(OH)_2D_3$ and its analogs.[27] Levels of 24-hydroxylase transcripts increased over a 24 h period but no further increase in TNF-RI mRNA was observed (Figure 4a,c). The above studies suggest that the potentiation of TNFα actions by vitamin D analogs is unlikely to be through enhanced recognition of the TNFα signal. We next sought to determine if vitamin D analogs may enhance autocrine production of TNFα. MCF-7 cell cultures were treated with ethanol vehicle or CB1093 (100 nM) for 1 – 24 h and level of induction of TNFα transcripts determined by RT – PCR. As with TNF-RI, an approximately 1.5 fold increase in the induction of TNFα transcripts was observed after 1 h of CB1093 treatment which was maintained for up to 4 h (Figure 4b). To further investigate whether pretreatment with vitamin D analogs may increase autocrine production of TNFα, MCF-7 cell cultures were treated with ethanol vehicle, CB1093 or TNFα for 5 days. Conditioned medium from these cultures was collected and analyzed in a cytotoxicity assay using the highly TNFα-sensitive cell line WEHI 164 clone 13. Medium

was conditioned between days 3–5 of treatment. To eliminate possible direct inhibitory effects of CB1093 present in conditioned medium on the cytotoxicity assay target WEHI cells, parallel cultures received the agents for days 0–3 of treatment only and then fresh medium without these additions was added for days 3–5 and TNFα-like bioactivity in 3–5 days conditioned medium assessed by cytotoxicity assay. No evidence of increased TNFα secretion in CB1093 treated cells was observed and levels of TNFα-like activity were below the detection limit of the bioassay (250 fg/ml, Table 1).

CB1093 promotes C_2-ceramide-induced cytotoxicity in MCF-7 cells

A possible mechanism by which vitamin D analogs may potentiate TNFα-induced cytotoxicity is by promotion of a common apoptotic pathway. Generation of ceramide through hydrolysis of sphingomyelin appears to play a role in TNFα-induced apoptosis. We next determined if pretreatment with CB1093 could modulate this step in the TNFα signaling pathway. MCF-7 cells were pretreated with CB1093 (50 nM for 48 h) and exposed to cell permeable C_2-ceramide (0.5–20 μM for 24 h) before DNA fragmentation assay. CB1093 augmented both the level of C_2-ceramide-induced intranucleosomal DNA fragmentation and loss of cell viability (Figure 5a,b).

CB1093 potentiates effect of TNFα on activation of $cPLA_2$

In some cells, TNFα-mediated killing is accompanied by activation of $cPLA_2$. Arachidonic acid generated as a result of $cPLA_2$ activation has been implicated in a signal transduction pathway resulting in cell death.[25,28] To investigate the possible role of $cPLA_2$ activation in the potentiation of TNFα effects by vitamin D analogs, MCF-7 cells were pretreated with CB1093 (50 nM for 48 h) and then labeled with [^{3}H]-AA (0.3 μCi/ml) for 18 h, washed and post-treated with TNFα (0–10 ng/ml) for a further 20 h. A dose related increase in the activation of $cPLA_2$, as assessed by [^{3}H]-AA release, was observed with TNFα which was potentiated when cells were pretreated with

Table 1 Effects of CB1093 and TNFα on MCF-7 cell viability and TNFα-like bioactivity in MCF-7 cell conditioned medium

	MCF-7 cell viability (NR uptake OD 550 nm)	Viability of WEHI cells incubated with MCF-7 CM % control^/TNF+
(a) Treated 0–5 days (CM 3–5 days)		
Control	1.134±0.14[a]	100.6±8.3/ND[a]
CB1093	0.345±0.049***	98.5±6.49/ND
TNF	0.952±0.068*	60.4±8.33***/1.3 pg/ml
TNF+CB1093	0.276±0.036***	61.1±7.0***/1.1 pg/ml
(b) Treated 0–3 days (CM 3–5 days)		
Control	1.167±0.10	95.3±7.2/ND
CB1093	0.268±0.052***	99.9±12.8/ND
TNF	0.861±0.081***	91.8±8.5/ND
TNF+CB1093	0.249±0.024***	93.9±6.3/ND

Medium conditioned by control (ethanol vehicle), CB1093 (50 nM) and TNFα (0.1 ng/ml) treated cells was assessed in a 20 h cytotoxicity assay using the highly TNF-sensitive cell line WEHI 164 cl 13. (**a**) MCF-7 cells were plated in 24 well plates as described in Materials and Methods. Cultures were treated for 5 days with CB1093, TNF or these agents in combination with a medium change on day 3. TNF-like bioactivity in 3–5 day conditioned medium was assessed by bioassay. (**b**) To eliminate possible direct inhibitory effect of CB1093 on the cytotoxic assay target WEHI cells, parallel cultures received the agents for days 0–3 of treatment and then fresh medium without these additions was added for days 3–5. TNF-like bioactivity in 3–5 day conditioned medium was assessed by bioassay. [a]Mean±S.E.M. of six replicate cultures. Significantly different from respective controls. ***$P<0.0001$, *$P<0.005$. ^control cultures incubated with non-conditioned medium +TNF concentration calculated from bioassay in relation to rhTNFα standard. ND=no TNF-like bioactivity detected. Detection limit of this assay is 250 fg/ml

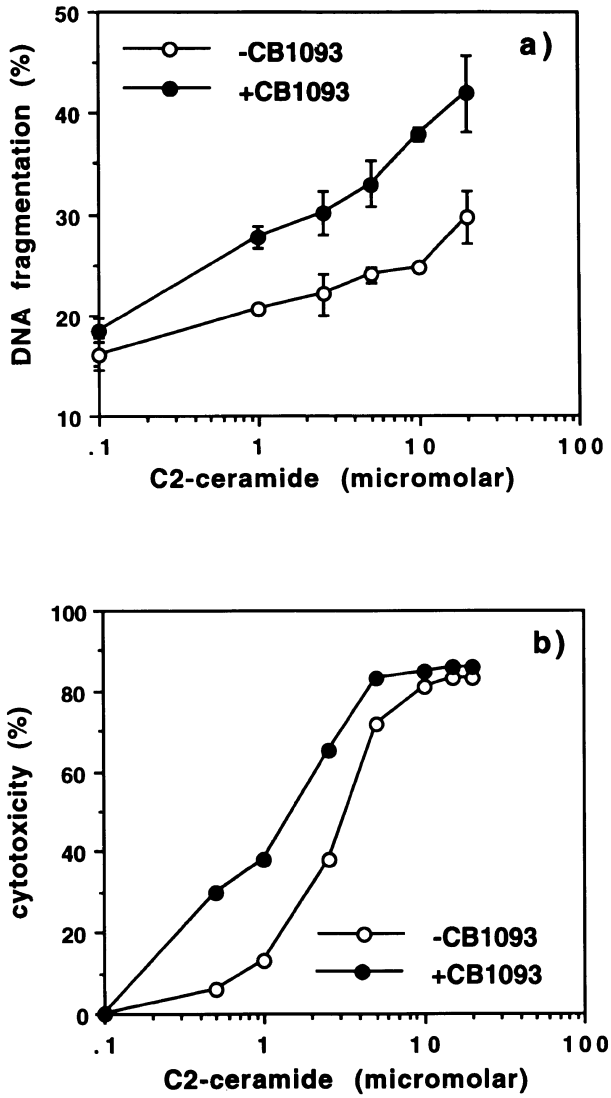


Figure 5 CB1093 promotes C_2-ceramide-induced apoptosis and loss of cell viability in MCF-7 breast cancer cells. (**a**) MCF-7 cells were labeled with [^{3}H-*methyl*]-thymidine (0.5 μCi/ml) for 24 h, washed twice and then exposed to cell permeable C_2-ceramide (0.5–20 μM) for a further 24 h and assessed for DNA fragmentation as described in Materials and Methods. (**b**) MCF-7 cells were pre-incubated with CB1093 (50 nM for 48 h), washed and then exposed to cell permeable C_2-ceramide (0.5–20 μM) for 24 h and assessed for cell viability by neutral red assay as described in Materials and Methods. Results are the mean±S.D. of three replicate estimations and are representative of three separate experiments

CB1093 (Figure 6a). The activation of $cPLA_2$ and its enhancement by CB1093 was inversely related to cell viability as determined by neutral red assay (Figure 6b). The relationship of $cPLA_2$ to potentiation of TNFα-induced cytotoxicity to CB1093 was determined by cotreating CB1093-primed cells with TNFα (0–10 ng/ml) and the specific $cPLA_2$ inhibitor $AACOCF_3$. Results showed that this inhibitor limited induced $cPLA_2$ potentiation of both CB1093 and TNFα in CB1093-primed MCF-7 cells (Figure 6a,b).

C_2-ceramide activation of $cPLA_2$ is enhanced by CB1093

Since it has been suggested that arachidonic acid released during $cPLA_2$ activation may act as a second messenger to activate other apoptosis associated enzymes such as sphingomyelinase leading to the generation of ceramide, we sought to identify the relationship of $cPLA_2$ activation to ceramide-induced apoptosis. To this end, MCF-7 cells were labeled with [^{3}H]-AA and then exposed to membrane permeable C_2-ceramide for 4 h and $cPLA_2$ activity assayed as previously described. Our results clearly show that incubation of MCF-7 cells with C_2-ceramide leads to a dose dependent activation of $cPLA_2$ (Figure 7a), suggesting that this enzyme may act down stream of ceramide generation in the apoptotic cascade. Activation

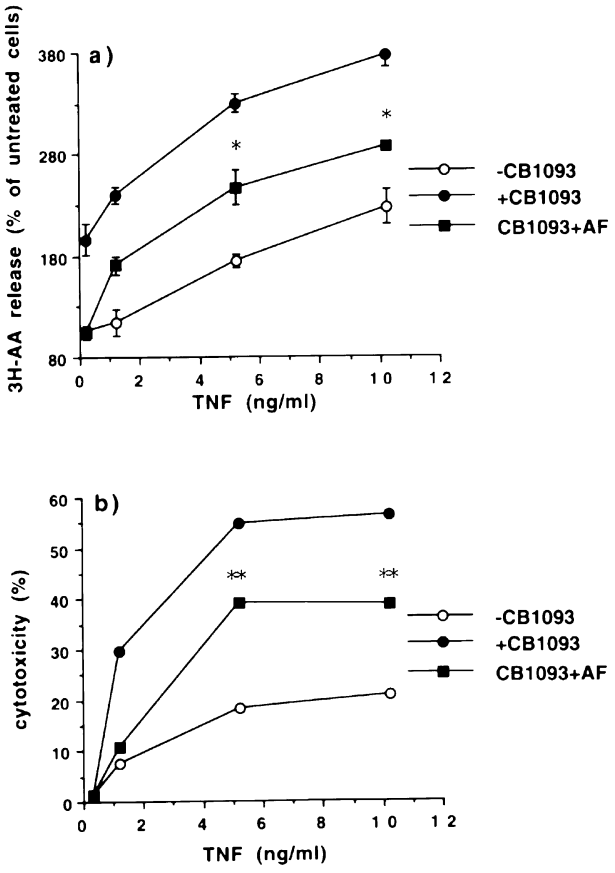


Figure 6 Effect of CB1093 and TNFα on activation of $cPLA_2$ in MCF-7 breast cancer cells. (**a**) MCF-7 cells were pretreated with CB1093 (50 nM for 48 h) and labeled with [^{3}H]-AA (0.3 μCi/ml) for 18 h, washed and post-incubated with or without 5 μM $AACOCF_3$ (AF) for 3 h and finally exposed to TNFα (0–10 ng/ml) for further 18 h. $CPLA_2$ activation was assessed by ^{3}[H]-AA release as described in Materials and Methods. (**b**) Replicate cultures were assessed for cytotoxicity by neutral red assay, as described in Materials and Methods. Statistical comparison between CB1093 and TNFα alone or CB1093 and TNFα plus $AACOCF_3$ was found to be significant at $^*P<0.05$ and $^{**}P<0.001$. Results are the mean±S.D. of three replicate estimations and are representative of three separate experiments

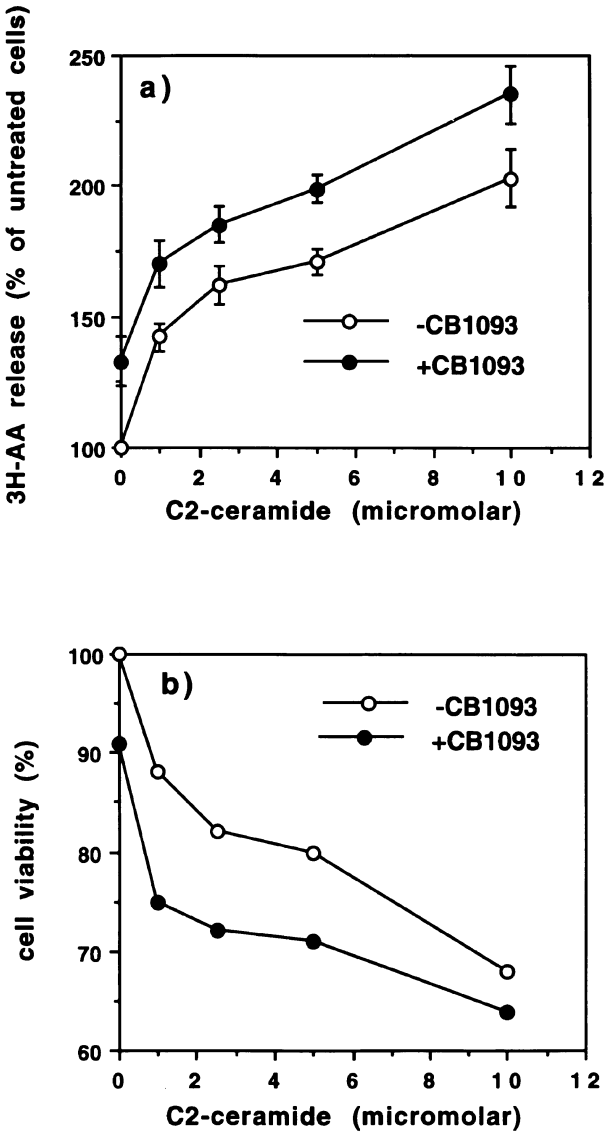
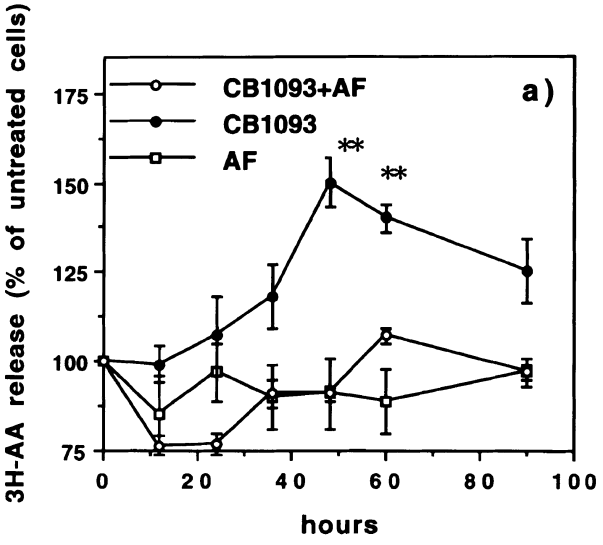


Figure 7 CB1093 promotes C_2-ceramide-induced activation of $cPLA_2$ and loss of cell viability in MCF-7 cells. (**a**) Cells were pretreated with CB1093 (50 nM for 48 h) and labeled with [^{3}H]-AA (0.3 μCi/ml) for 18 h, washed and exposed to C_2-ceramide (0–20 μM) for further 5 h. $CPLA_2$ activation was assessed by [^{3}H]-AA release as described in Materials and Methods. (**b**) Replicate cultures were assessed for cell viability by MTS assay, as described in Materials and Methods. Results are the mean±S.D. of three replicate estimations and are representative of three separate experiments

of $cPLA_2$ in response to C_2-ceramide is more rapid than with TNFα being evident 4 h after cells are exposed to C_2-ceramide in comparison to 20 h with TNFα. Furthermore, cells primed for 48 h with CB1093 show enhanced $cPLA_2$ activation in response to ceramide. Cell viability was inversely correlated with $cPLA_2$ activation (Figure 7b).



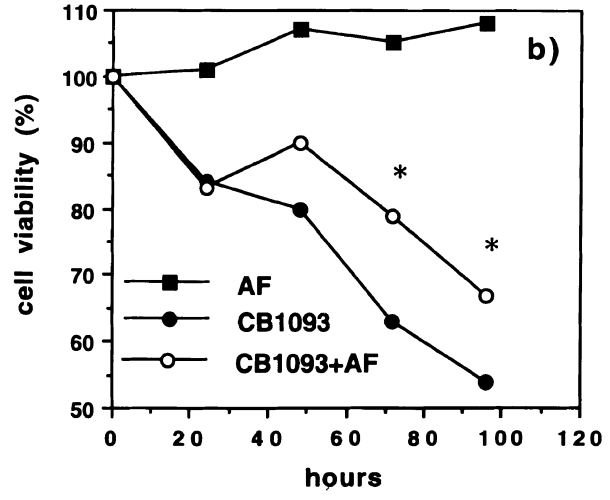


Figure 8 (**a**) CB1093 induces $cPLA_2$ activation in MCF-7 cells. MCF-7 cells were labeled with [^{3}H]-AA (0.3 μCi/ml) for 18 h, washed extensively and incubated with or without specific inhibitor $AACOCF_3$ (5 μM) for 3 h and then treated with CB1093 (50 nM for 0–96 h). $cPLA_2$ activation was assessed by [^{3}H]-AA release as described in Materials and Methods. Statistical comparison between CB1093 alone and CB1093 plus AF was found to be significant at $^{**}P<0.001$. (**b**) Effect of $AACOCF_3$ on CB1093-induced loss of cell viability in MCF-7 cells. Replicate cultures were assessed for cell viability by MTS assay, as described in Materials and Methods. Statistical comparison between CB1093 alone and CB1093 plus AF was found to be significant at $^{*}P<0.005$. Results are the mean±S.D. of three replicate estimations and are representative of three separate experiments

Loss of cell viability in MCF-7 cells treated with CB1093 is associated with $cPLA_2$ activation

To determine if activation of $cPLA_2$ could be involved in the induction of MCF-7 cell apoptosis induced by vitamin D analogs in the absence of TNFα, activity of this enzyme was determined in cells treated from 2–5 days with 50 nM of CB1093. Results show that treatment with CB1093 alone increases $cPLA_2$ activity by 2 days of treatment and that this activation is prevented by the specific $cPLA_2$ inhibitor $AACOCF_3$ (Figure 8a). Furthermore, coincubation of cells with CB1093 and $AACOCF_3$ led to partial protection against the loss of cell viability induced by CB1093 alone (Figure 8b). This protection was evident from 50 h of treatment, the time at which $cPLA_2$ activation was observed. These findings suggest that activation of $cPLA_2$ may play a role in vitamin D-induced apoptosis in MCF-7 breast cancer cells.

Discussion

The biologically active form of vitamin D, the nuclear hormone 1,25-dihydroxyvitamin D_3, is an important regulator of cell growth, differentiation and apoptosis. Actions of this hormone are mediated by activation of the vitamin D receptor (VDR), a member of the super family of nuclear receptors acting as ligand-inducible transcription factors.[29] Synthetic analogs of vitamin D have been shown to display antiproliferative effects on breast cancer cells both *in vivo* and *in vitro*. EB1089 has been demonstrated to induce MCF-7 cell apoptosis *in vitro*[10,12,30] and *in vivo* when these cells are grown as tumor

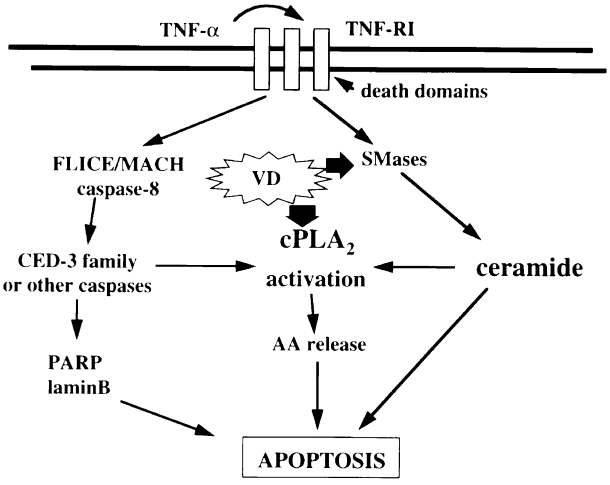


Figure 9 Proposed scheme for interaction of vitamin D analogs with pathway for TNFα-induced apoptosis in breast cancer cells. The 55 kDa TNF receptor initiates apoptosis via formation of a death domain adaptor protein complex that links downstream to neutral sphingomyelinase[22] and caspase activation leading to PARP cleavage and apoptosis. Activation of $cPLA_2$ may be promoted via ceramide signaling[20] and/or via CED-3 caspase activation.[25] Arachidonic acid, generated by $cPLA_2$ activation, can lead to loss of membrane integrity and cell death. It is suggested that vitamin D analogs, through altered transcription of target genes, increase generation of ceramide and $cPLA_2$ activation such that apoptosis can be initiated in response to sub-optimal concentrations of TNFα

xenografts in nude mice.[31] The novel vitamin D analog CB1093 has been shown to display potent antitumor effects *in vivo* and *in vitro* and its effects on induction of apoptosis are evident at concentrations approximately tenfold lower than EB1089.[10] Furthermore, induction of apoptosis has been reported to be accompanied by decreased bcl-2 protein expression.[10] In the present study we demonstrate that preincubation of MCF-7 cells with vitamin D analogs potentiates the effects of TNFα on induction of apoptosis. Enhanced responsiveness was demonstrated by a reduction in time of treatment required to observe cell death and the dose of cytokine at which substantial cytotoxicity was observed. Rocker *et al*.[49] reported a similar time course of cytotoxicity in response to cotreatment with $1,25(OH)_2D_3$ and TNFα in a TNFα-sensitive MCF-7 subclone. In contrast, no significant cytotoxicity with anti-Fas treatment was detected in both control and vitamin D analog pretreated cells. This is in agreement with other studies demonstrating little effect of anti-Fas in wild-type MCF-7 cells although apoptosis is achieved in cells over expressing Fas.[32] Using videomicroscopy we observed typical morphological changes associated with apoptosis, such as shrinkage and blebbing, in MCF-7 cells treated with CB1093 alone or in combination with TNFα, but these changes were less evident in cells treated with TNFα alone. These results correlated well with data obtained from DNA fragmentation analysis where 40% DNA fragmentation was detected after 48 h exposure of CB1093-primed cells to TNFα, while the cytokine alone was able to induce only 25% DNA fragmentation for the same period of time. It has been reported that TNFα-induced cytotoxicity in MCF-7 cells is not accompanied with typical apoptotic morphological changes, possibly due to functional deletion of the caspase-3 gene.[33] In this regard we suggest that pretreatment with vitamin D analogs may potentiate TNFα-mediated cell death in MCF-7 cells by targeting cleavage of key proteins important for maintenance of cell architecture.

Possible mechanisms by which vitamin D analogs enhance responsiveness to TNFα may involve increased signal recognition, decreased expression of proteins which lead to TNFα resistance or the promotion of a common apoptotic pathway. It has been recently shown that differences in susceptibility of TNFα-induced apoptosis among MCF-7 cells variants may be explained by differences in TNF-RI expression.[34] We have been unable to observe substantial increases in the expression of the receptor for TNFα in response to vitamin D analogs. Using RT–PCR, we have evaluated transcripts for TNF-RI in MCF-7 cells treated with CB1093 and no marked increases were observed over a time course of 1–24 h. In contrast, treatment with the vitamin D analog produced a marked induction of the 24-hydroxylase gene which previously has been shown to be a primary vitamin D responding gene.[27] Although there are indications that the TNFα gene is also a target gene for $1,25(OH)_2D_3$,[35] promotion of apoptosis by vitamin D analogs does not appear to be related to increased endogenous production of TNFα in MCF-7 cells, as we have been unable to detect increased elaboration of TNFα-like bioactivity in conditioned medium or substantial increases in the level of TNFα transcripts in response to CB1093.

Another possible mechanism by which vitamin D analogs may potentiate TNFα-induced cytotoxicity is by promotion of a common apoptotic pathway. Several intracellular pathways have been demonstrated to be involved in the TNFα-initiated cytotoxic process, including reduction of glutathione levels, free radical generation[36] and activation of the caspase system.[37] TNFα-mediated apoptosis in MCF-7 cells has been reported to be associated with a several fold accumulation of ceramide production, starting at 10–12 h and reaching fourfold by 24 h.[38] Furthermore, it has been documented that inability of TNFα to induce ceramide formation and $cPLA_2$ activation leads to resistance to TNFα-mediated cell death in MCF-7 cells.[39] Our present findings have shown that vitamin D analogs increase responsiveness of MCF-7 cells to C_2-ceramide in terms of loss of cell viability, DNA fragmentation and ceramide-induced activation of $cPLA_2$. For all three cellular functions the dose-response curve to C_2-ceramide was shifted to the left in CB1093 primed cells. Of interest are reports that $1,25(OH)_2D_3$ promotes generation of ceramide (an increase of 40–70% after 1–2 h of treatment) which plays an important role in regulation of cell proliferation in HaCaT cells and cell differentiation in HL-60 cells respectively.[35,40] If the same is true in MCF-7 cells, then pretreatment with vitamin D analogs may increase intracellular ceramide to a critical level whereby active cell death can be initiated in response to sub-optimal concentrations of TNFα.

Our studies show that TNFα activates $cPLA_2$ in MCF-7 breast cancer cells. Activation of this enzyme has been shown to be essential for TNFα action on L929 cells as TNFα resistant mutants lacking $cPLA_2$ expression displayed TNFα sensitivity following exogenous expression of $cPLA_2$.[41] Inhibition of $cPLA_2$ by antisense strategies rendered melanoma cells resistant to TNFα-mediated cytotoxicity.[28] A specific inhibitor of $cPLA_2$($AACOCF_3$) inactivates $cPLA_2$ by competitive binding to the active site of this enzyme.[42] Wissing *et al*.[25] showed that $AACOCF_3$ inhibits TNFα-induced cytotoxicity in MCF-7 and WEHI cells further supporting the suggestion that $cPLA_2$ is an essential part of the TNFα-induced death pathway. Our results show that preincubation with $AACOCF_3$ prevents CB1093 potentiation of $cPLA_2$ activation. The mechanism by which $cPLA_2$ mediates apoptosis is still unclear. Interestingly, it has been shown recently that activation of $cPLA_2$ in TNFα-induced apoptosis is caspase dependent.[25] This group demonstrated that a specific inhibitor of the caspase-3 family (Ac-DEVD-cho) inhibited TNFα-induced $cPLA_2$ cleavage and AA release. Using a broad spectrum caspase inhibitor (z-VAD-fmk) we have confirmed that TNFα-induced $cPLA_2$ is caspase dependent. However our preliminary data show that z-VAD-fmk does not protect against CB1093-induced loss of MCF-7 cell viability, suggesting that caspases may not be involved in this signaling pathway (data are not shown). More intriguing data have been recently reported by Luschen *et al*.[43] who have demonstrated that the presence of multiple sites of $cPLA_2$ cleavage by several caspases may generate a variety of cleavage products, which may have different roles in inflammation or apoptosis. The observation that $cPLA_2$ activation is caspase-dependent has prompted the

suggestion that it must act in the final execution step of apoptosis and that $cPLA_2$-mediated release of AA may disrupt the integrity of various cellular membranes.[25] An alternative suggestion is that the released AA may act as a second messenger and activate other apoptosis-associated enzymes such as sphingomyelinase.[25] Our observation that exogenous ceramide itself promotes activation of $cPLA_2$ in a dose dependent manner makes this suggestion less likely. Alternatively the localization of the sphingomyelin pool in different cell compartments and gradual accumulation of ceramide in TNFα-mediated cell death in MCF-7 cells, support the suggestion that ceramide formation might be both upstream and downstream of the caspase-3 family or acts in different stages of apoptosis.[44] Ceramide is known to induce cell differentiation, cell cycle arrest or apoptosis depending on cell type and activation of other signal transduction pathways. Interestingly, vitamin D derivatives appear to modulate TNFα signaling to produce two distinct cell type specific effects. It has been reported that $1,25(OH)_2D_3$ protects MG-63 osteoblasts from the cytotoxic effects of TNFα[45] and U937 leukemic cells induced to differentiate by pretreatment with EB1089 did not undergo apoptosis when exposed to TNFα while uninduced control culture were susceptible to the cytotoxic effects of the cytokine.[46] Of considerable interest is the recent observation in U937 cells that pretreatment with $1,25(OH)_2D_3$ inhibits both TNFα-induced apoptosis and activation of $cPLA_2$ in response to TNFα.[47] Our present findings whereby vitamin D analogs potentiate TNFα-induced activation of $cPLA_2$ in MCF-7 cells suggests that differential effects on $cPLA_2$ activation may be related to the ability of vitamin D derivatives to modulate TNFα-induced apoptosis in cell-type specific manner. Furthermore we have established a system where vitamin D analogs do not enhance the susceptibility of MCF-7 cells to undergo apoptosis. In agreement with recent findings that $1,25(OH)_2D_3$ does not affect cytotoxicity induced by etoposide in MCF-7 cells,[48] we have found that CB1093-primed MCF-7 cells do not show enhanced sensitivity to etoposide-induced apoptosis and furthermore, etoposide does not promote $cPLA_2$ activation (data are not shown).

In addition to promoting TNFα and ceramide-induced apoptosis, vitamin D derivatives alone induce $cPLA_2$ activation and active cell death in MCF-7 cells indicating that $cPLA_2$ may be involved in vitamin D-induced apoptosis. However, while substantial cell death is detected in vitamin D pretreated cells after 18 h treatment with TNFα, effects of vitamin D analogs alone on apoptosis are only observed after 3–5 days of treatment. This suggests a requirement for altered expression of genes in response to these analogs and increased (or decreased) synthesis of bioactive proteins which ultimately result in the demise of the cells by apoptosis. Further studies are required to identify the nature of these primary target genes for vitamin D.

In summary, the results of our study indicate that synthetic analogs of vitamin D potentiate the responsiveness of MCF-7 breast cancer cells to TNFα-induced apoptosis. The relation of our results to *in vivo* efficacy of TNFα in combination with vitamin D analogs warrants further investigations. Our findings further suggest that vitamin D analogs share a common pathway leading to cell death which involves $cPLA_2$ activation and/or ceramide generation (Figure 9) such that apoptosis can be initiated in response to sub-optimal concentrations of TNFα. The relationship of $cPLA_2$ activation and ceramide generation to vitamin D-induced apoptosis in other cell types requires further investigation.

Materials and Methods

Reagents

EB1089 (22,24-diene-24α,26α,27α-trihomo-1α,25-dihydroxyvitamin D_3) and CB1093 (20-epi-22(S)-ethoxy-23yne-24α,26α,27α-trihomo-1α,25-dihydroxyvitamin D_3) were gifts from Dr. Lise Binderup Leo Pharmaceutical Products, Denmark. The vitamin D compounds were dissolved in ethanol and stored at $-20^{\circ}C$. For use in experiments the ethanol concentration did not exceed 0.1% in tissue culture medium. Human recombinant TNF alpha (TNFα), C_2-ceramide, anti-Fas and $cPLA_2$ inhibitor Arachidonyl trifluoromethylketone ($AACOCF_3$) were purchased from Calbiochem, UK. [^{3}H-*methyl*]-thymidine (80.0 Ci/mmol) was purchased from Amersham and 5.6.8.9.11.12.14.15-[^{3}H]-arachidonic acid (240 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). Tissue culture media (RPMI-1640, DMEM) were obtained from Life Technologies (Paisley, Scotland).

Cell cultures

The MCF-7 human breast cancer cell line was maintained in Dulbecco's modification of Eagle's medium DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum (FCS). In some experiments phenol-red free DMEM medium supplemented with 5% charcoal stripped FCS was used with similar results.

Cell viability and DNA fragmentation assays

Neutral red assay Neutral red is a vital dye that accumulates in the lysosomes of living, uninjured cells. Cytotoxicity of TNFα in control and vitamin D analog pretreated cultures was determined by the method of Rocker *et al*.[49] Briefly MCF-7 cells were seeded into 24 well plates (Nunc, Oxford, UK) at a density of 2×10^4 cells/well in 5% FCS, DMEM and left to adhere for 4–5 h after which medium containing vitamin D analogs was added. Following a preincubation period of 24–67 h, fresh medium containing the analogs with and without TNFα (0.2–20 ng/ml) was added and cultures were incubated for a further 18–48 h. In some experiments, anti-Fas (250 ng/ml) was added in place of TNFα. In other experiments, cells were switched to serum-free medium (RPMI-1640, bovine serum albumin 0.5 mg/ml and transferrin 0.01 mg/ml) and C_2-ceramide was added. At the end of the incubation period, medium was removed and cells were incubated with neutral red solution (from Sigma, 40 μg/ml in phenol-red and serum free DMEM) for 2 h at 37°C. After removal of the neutral red solution, wells were rinsed once with 1 ml 4% formal saline containing 0.5% $CaCl_2$. Plates were inverted on paper towel to drain and 200 μl of elution fluid (1% acetic acid in 50% ethanol) was added. Following incubation at room temperature for 30 min with gentle shaking, absorbance at 550 nm was determined using a Titertec plate reader. The cytotoxic effect of TNFα was calculated as previously described.[49]

MTS assay Cell viability was determined by MTS dye-reduction assay measuring mitochondrial respiratory function.[50] MCF-7 cells were plated in 96 well microtiter plates and treated with compounds of

interest for various lengths of time. Cells were incubated with MTS dye (2 mg/ml, 20 μl/well) for 4 h, and solubilized with 10% SDS at room temperature for 16 h. Absorbance was read in a Titertek plate reader at 492 nm. The absorbance is directly related to viable cell number.

DNA fragmentation assay MCF-7 cells were incubated with [^{3}H-*methyl*]-thymidine (0.5 μCi/ml) for 24 h to label DNA and then washed before exposure to the indicated agents. Cells were lysed and [^{3}H-*methyl*]-thymidine incorporated into both soluble and unfragmented DNA was determined by liquid scintillation counting using the formula: per cent fragmented DNA=100 × (fragmented/fragmented+intact chromatin) as previously described.[51]

Videomicroscopy In order to further validate the time course of induction of apoptosis by combinations of TNFα and vitamin D analogs, videomicroscopy was used to directly observe cell death. MCF-7 cells were plated in T25 flasks and treated for 42 h with ethanol vehicle (control) or 25 nM CB1093 and then TNFα (10 ng/ml final concentration) was added to the flasks. Four hours after addition of TNFα typical fields from CB1093/TNFα and control/TNFα treated cultures were photographed over a 48 h period at 1/150 normal speed with a time-lapse videorecorder. After this period, typical fields from control and CB1093 treated cultures in the absence of TNFα were similarly photographed.

Detection of TNFα, TNF-RI and 24-hydroxylase mRNA by semi-quantitative PCR

MCF-7 cells were maintained in phenol red-free DMEM supplemented with 2.5% charcoal-treated FCS. Cultures at 70% confluence were treated with vitamin D analogs or ethanol vehicle (0.1%) for 1–24 h, then collected and total RNA isolated with Trizol Reagent (Life Technologies, Paisley, Scotland). First strand cDNA synthesis was performed on 2 μg total RNA. Reverse transcription was carried out at 37°C for 60 min in a final volume of 40 ml in the presence of 0.25 μM oligo dT15-primer, 0.5 mM dNTPs, 5 mM DTT, 1 U RNasin, 5 U MMLV-RT (LIfe Technologies) and 1 × first strand buffer (0.05 M Tris-HCl, pH 8.3, 0.075 M KCl, 3 mM $MgCl_2$). The cDNA initially generated was precipitated and then dissolved in water to give a final volume of 200 ml and of this 5 ml was taken for use in PCR. The following oligonucleotide primers were used to represent: TNF-α+; 5′-TCTTGC-ACAGTGGACCGGGA-3′; TNFα−; 5′-CACCGTTGGTAGCGATACAT-3′; TNF-RI-; 5′-ATTTGCTGTACCAAGTGCCACAAAGGAACC-3′; TNF-RI; 5′-GTCGATTTCCCACAAAAACAATGGAGTAGAGC-3′; 24-hydroxylase+; 5′-CTGCTGCAGATTCTCTGGA-3′; 24-hydroxylase−; 5′-ATGATGAAGTTCACAGCTTC-3′ and β_2 microglobulin+; 5′-CCCCCACTGAAAAAGATGAGTATGCCTG-3′; β_2 microglobulin−; 5′-CCTGTGGAGCAACCTGCTCAGATACCATC-3′.

Primers were 5′ end labeled with ^{32}PdATP. The following PCR conditions were used for TNFα, 24-hydroxylase and β_2 microglobulin; 94°C for 5 min, 94°C for 1 min, 58°C for 2 min, 72°C for 300 s for 40 cycles then 94°C for 10 s, 58°C for 1 min, 72°C for 30 s for 30 cycles and only 22 cycles for β_2 microglobulin, then 72°C for 10 min. For TNF-RI an annealing temperature of 55°C was used. Amplified TNFα, TNF-RI, 24-hydroxylase or β_2 microglobulin mRNA was resolved on a 5% nondenaturing polyacrylamide gel in 0.5 TBE [45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.3)]. Following autoradiography, the bands representing the radioactive free probe and the radioactive TNFα, TNF-RI, 24-hydroxylase/β_2 microglobulin probes were localized, excised and assayed directly by scintillation counting. The amount of TNFα, TNF-RI and 24-hydroxylase mRNA was normalized to β_2 microglobulin mRNA and fold induction of response was calculated as the ratio of ligand stimulated cells to that of vehicle treated controls.

Determination of biological activity in culture supernatants

MCF-7 cells were plated in 24-well plates and treated with ethanol vehicle, 50 nM CB1093, 0.1 ng/ml TNFα or CB1093 together with TNFα for 3 days. Media were removed, the cells washed three times in serum-free medium and then 1 ml of either medium containing CB1093 and/or TNFα at these same concentrations or fresh medium without these agents was added to the cultures. Media were conditioned for 2 days before being aspirated, centrifuged at 8000 × g and supernatants stored at −20°C before assay. TNFα bioactivity was quantified without further dilution in a 22 h cytotoxicity assay using WEHI 164 clone 13 cells as previously described.[52] The detection limit of this assay was 250 fg/ml.

Activation of cPLA$_2$

Measurement of [^{3}H]-arachidonic acid (AA) release from MCF-7 breast cancer cells was determined as described by Jaattela *et al*.[32] Briefly, MCF-7 cells seeded in 24 well plates (2×10^4/well in 0.5 ml) and primed with or without CB1093 for 24–48 h were labeled with 0.3 μCi/ml 5.6.8.9.11.12.14.15-[^{3}H]-arachidonic acid and incubated at 37°C for the final 18 h of treatment. The unincorporated [^{3}H]-AA was removed by washing three times with serum-free medium. Fresh medium was added containing graded concentrations of TNFα (0.1–20 ng/ml) or vehicle for 22 h. In some experiments cells were switched to serum-free medium and C_2-ceramide (0.5–20 μM) was added for 5 h. In experiments using $AACOCF_3$ inhibitor, cells were first pretreated for 3 h with $AACOCF_3$ and then exposed to the drugs. Media were removed, centrifuged at 2000 × g for 5 min and 0.5 ml of supernatant processed for liquid scintillation counting. Cells were removed by trypsinization, and cell pellets solubilized in 0.5 M sodium hydroxide and assessed for radioactivity. The percentage of release of [^{3}H]-AA was calculated by the formula [S/(S+P)] × 100 where S and P represent radioactivity detected in 0.5 ml of supernatant and solubilized cell pellet respectively. Non enzymic release of [^{3}H]-AA from prelabeled cells, frozen at −80°C and thawed at room temperature, was less than 10% in all experiments.[47] Results were expressed as a per cent of [^{3}H]-AA release in nontreated cells (accepted as 100%).

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test or analysis of ANOVA using the Stat-view-4 software package (Apple Macintosh). $P<0.05$ was considered statistically significant.

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