Calcitonin decreases the adherence and survival of HEK-293 cells by a caspase-independent mechanism

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Abstract

We recently reported that calcitonin (CT) can profoundly inhibit the growth of HEK-293 cells transfected with the human calcitonin receptor (hCTR). We also obtained preliminary evidence that suggested a role for CT in cell survival, and in the present study we have investigated the pro-apoptotic action of CT, which we observe in conditions of low serum concentration. Under these conditions, we have found that CT treatment of HEK-293 cells stably transfected with the insert-negative form of the human CTR (HR12 cells) caused a time-dependent decrease in cell number associated with loss of cellular attachment. Loss of cellular adherence in CT-treated cultures caused programmed cell death, as shown by Annexin V staining of cells, failure of cells to exclude Trypan Blue dye, condensation and cleavage of nuclear DNA, and appearance of hypodiploid cells in fluorescence-activated cell sorting (FACS) analysis. The accumulation of non-adherent cells and cell death was concomitant with increased intracellular activity of caspase-3. However, inhibition of caspase activation in HR12 cells did not prevent CT-mediated loss of attachment and did not maintain the viability of non-adherent cells, indicating that caspase activation accompanied, but was probably not the cause of, the loss of cell viability. Neither the effects of CT on cell survival nor the activation of caspase-3 were observed in serum-replete conditions, suggesting that serum-derived factors provide protection of cells from CT-induced apoptosis. The inhibitory effects of CT on cell growth were found previously to be related to activation of Erk1/2 MAP kinase. In the present experiments, it was found that the Erk1/2 inhibitor, PD 98059, inhibited the CT-induced loss of cellular adherence and the consequent reduction in cell numbers. These results demonstrate that CT can negatively affect cell survival and they identify roles for cell adherence and MAP kinase activation in this process.

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Introduction

Calcitonin (CT) is a 32 amino acid peptide hormone of thyroidal origin, whose main recognised physiological role is the inhibition of bone resorption by acting directly on osteoclasts (Chambers & Magnus 1982). However, both CT and its receptors (CTR) have also been identified in a large number of other cell types and tissue sites (Kuestner et al. 1994, Jagger et al. 1999, Sexton et al. 1999), suggesting roles for the CT/CTR system distinct from those involving calcium homeostasis. In particular, there is evidence consistent with the involvement of CT in cell growth and differentiation and in tissue development and remodelling.

CT appears to be important for both blastocyst implantation (Zhu et al. 1998) and for development of the early blastocyst (Wang et al. 1998). In addition, embryonic expression of the mouse CTR, and of a lacZ construct driven by the CTR promoter (Jagger et al. 1999, 2000), suggest that CTR may play important roles in morphogenesis. The observation by Ng et al. (1983) that treatment of human breast cancer cells with CT inhibited cell proliferation, together with the reported mitogenic action of CT in certain prostate cancer cell lines (Shah et al. 1994), supports a role for CT in the modulation of cell proliferation.

The human embryonal kidney (HEK) cell line, HEK-293, has proven useful as an expression system for many proteins, including receptors. We have described the transfection of HEK-293 cells with several isoforms of the human CTR (hCTR) (Evdokiou et al. 1999), including the predominant insert-negative splice variant that lacks a 16 amino acid insert in the putative first intracellular loop (Kuestner et al. 1994). We found that CT profoundly
inhibited the growth of HEK-293 cells over-expressing the insert-negative hCTR (HR12 cells; Evdokiou et al. 1999). CT caused an accumulation of cells in the G2 phase of the cell cycle, associated with a prolonged increase in p21WAF1/CIP1 expression and a sustained activation of the p42/44 MAP kinase proteins (Raggatt et al. 2000). We also obtained preliminary evidence that suggested a role for CT in cell survival, which appeared to be dependent on the cell growth conditions (Evdokiou et al. 1999).

In the present study we have further investigated the effect of CT on cell survival under low serum conditions. We have found that CT treatment of HEK-293 cells, stably transfected with the insert-negative form of the hCTR, caused a time-dependent decrease in cell number, which was concomitant with, but independent of, increased intracellular activity of caspase-3. Loss of cell viability was associated with loss of cellular attachment, indicating that CT treatment caused cell death by anoikis.

Materials and Methods

Salmon CT (sCT) was from Peninsula Labs Inc. (Belmont, CA, USA), U1026 was from Tocris Cookson Inc. (Ellison, MO, USA) and PD-98059 and LY-294002 were from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The tetrapeptide caspase inhibitors, z-VAD-fmk and Boc-A-fmk, were purchased from Calbiochem (Alexandria, NSW, Australia).

Cell culture

The cell line designated HR12, which has been transfected to stably over-express the insert-negative isoform of the hCTR, has been described previously (Evdokiou et al. 1999). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (ICN Biomedicals Inc., Aurora, OH, USA) supplemented with 10% heat inactivated fetal bovine serum (FCS) (Multi Ser, Trace Biosciences Pty Ltd, Castle Hill, NSW, Australia), 2 mM glutamine, 100 U/ml penicillin, and 0·1 mg/ml streptomycin. HR12 cells were maintained in 200 µg/ml G418 (Gibco BRL, Glen Waverley, Vic., Australia), which was removed prior to commencement of experiments. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

Cell growth analysis

To determine the effect of CT or other agents on cell proliferation or survival, cells were plated in 12- or 24-well culture plates in 10% serum-containing media, at 2 × 10⁴ cells/well and allowed to attach and spread for 48–72 h before experiments. Media were then changed and experiments were initiated by the addition of CT or other agents for the times indicated, either under conditions of optimal mitogenic stimulus (10% FCS), low serum (0·5%) or serum-free conditions. Some experiments were performed in serum-free medium, using Biorich 1 medium from ICN Biomedicals, Inc., which contains no added growth factors or protein. For determination of cell proliferation, cells were harvested by trypsinisation and counted manually using a haemocytometer. Cell viability was determined by Trypan Blue exclusion.

Caspase activity assay

To assess the ability of sCT to induce caspase-3 activation, cells were plated in 48-well plates at 2 × 10⁴ cells/well in the appropriate culture conditions. Seventy-two hours after plating, cells were treated as described, for the times indicated. Cells were lysed using 100 µl caspase lysis buffer (5 mM EDTA, 5 mM Tris–HCl and 5% NP40), centrifuged for 3 min, and the supernatants were stored at −70°C. To determine caspase-3 activity, a caspase-3 fluorogenic substrate (Ac-DEVD-AFC) (Kamiya Biomedical Company, Seattle, WA, USA) was incubated with lysate in a 96-well plate format. Twenty microlitres of sample were mixed with 200 µl caspase assay buffer (50 mM HEPES, 300 mM sucrose, 1·5 mM CHAPS, 100 mM dithiothreitol, 2·5 µM caspase-3 fluorogenic substrate (Ac-DEVD-AFC), pH 7·4) and incubated for 5 h at room temperature. Fluorescence was quantified (Exc 400, Emis 505) in a Perkin Elmer luminescence spectrometer LS50B. Caspase activity was then normalised for the protein content of each sample and expressed as a proportion of the caspase activity recorded for the untreated cells.

Protein assay

A colorimetric BCA assay (Pierce, Rockford, IL, USA) was used to determine the amount of protein in each caspase sample. Briefly, a 4 mg/ml solution of BSA was used to establish a standard curve between 0 and 40 µg/ml. From each caspase sample 7·5 µl were aliquoted into a 96-well plate (Costar, Cambridge, MA, USA). Two hundred microlitres of protein assay reagent (50 parts of reagent A to 1 part of reagent B) were used to determine the amount of protein in each sample and expressed as a proportion of the caspase activity recorded for the untreated cells.
was read on a microtitre plate reader (Dynatech) at 570 nM. A standard curve was used to determine the amount of protein in each sample.

**DNA fragmentation**

Cells were harvested by washing twice with PBS, after 72 h in the presence or absence of 10 nM sCT, and incubated overnight at 37 °C in lysis buffer containing 10 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 1% SDS and 200 µg/ml proteinase K. DNA was extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and then precipitated in ethanol. Samples were electrophoresed in a 1:2% agarose gel, stained with ethidium bromide and visualised under UV light.

**DAPI staining of nuclei**

Cells were seeded on plastic chamber slides and treated with CT as indicated. After two washes with PBS, cells were fixed in methanol for 5 min, washed again with PBS, and incubated with 0.8 mg/ml 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche Diagnostics, Castle Hill, NSW, Australia) in PBS for 15 min at 37 °C. After several washes in PBS, the coverslips were mounted on PBS/glycerin. DAPI staining was visualised by fluorescence microscopy.

**Cell cycle analysis by FACS**

Cells were removed from culture dishes by trypsinisation, collected by centrifugation, resuspended in PBS and then fixed in ice-cold 70% ethanol. Cells were then washed in PBS and resuspended in a solution containing 0.1% Triton X-100 in PBS, 200 µg/ml DNase-free RNaseA (Sigma Co.), 20 µg/ml propidium iodide (Sigma Co.) and incubated for 20 min at 37 °C. The stained nuclei were analysed using a flow cytometer (Epics Profile, Coulter). Cell cycle distribution was based on 2 N and 4 N DNA content.

**Annexin V staining**

In some experiments apoptosis was determined by staining with fluorescent labelled Annexin V-PE and 7-amino actinomycin (AV/7 AAD) staining, which provides a sensitive early measure of apoptosis (Vermes et al. 1995). Briefly, floating non-adherent and adherent cells that were trypsinised were collected by centrifugation and resuspended in PBS at a concentration of 1 × 10^6 cells/ml. Five microlitres Annexin V-PE and 5 µl 7 AAD (Pharmingen, Sydney, NSW, Australia) were added to 100 µl of the cell suspension by gentle vortexing. Cells were incubated for 15 min at room temperature in the dark, before the addition of 400 µl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and were analysed by flow cytometry (Epics Profile, Coulter). The percentage of cells that were either Annexin V-positive and 7 AAD negative (AV⁺/7 AAD⁻; i.e. apoptotic cells) or Annexin
V-positive and 7 AAD-positive (AV+/7 AAD+; i.e. end stage apoptotic or necrotic cells) was calculated using the Coulter Epics software.

Statistical analysis

The results were analysed by either a Student’s \( t \)-test or one-way analysis of variance, as indicated. Where required, a Tukey’s Post Hoc test was used to determine the level of significance.

Results

CT induces apoptosis in CTR transfected HEK-293 cells under low serum conditions

We have reported that CT profoundly inhibits the growth of HEK-293 cells transfected with the insert-negative isoform of the hCTR (HR12) (Evdokiou et al. 1999). Growth suppression under conditions of optimal mitogenic stimulus (10% FCS) was due to a cytostatic response to CT treatment, as shown in Fig. 1A, with no evidence of cell death (Evdokiou et al. 1999). However, when cell proliferation was observed under conditions of reduced serum (0.5% FCS), treatment with 10 nM sCT resulted in a reduction of cell number to below the starting cell number added to the wells (Fig. 1B). After 72-h incubation with CT, only about 50% of the cells remained, indicating the ability of CT to induce cell death under low serum conditions.

It was observed that CT treatment of HR12 cells in 0.5% serum-containing medium resulted in a rounded morphology of cells and eventual loss, by day 3, of cell attachment to the culture dish. This is demonstrated in Fig. 2, which shows that CT treatment resulted in a loss of adherent cells (Fig. 2A) and an increased number of non-adherent cells (Fig. 2B) at day 3. Low numbers of non-adherent cells only were seen in untreated control cultures. Trypan Blue dye exclusion was used as a marker of cell viability and showed that almost 100% of both control and CT-treated adherent cells were viable (Fig. 2C). In contrast, CT-treated non-adherent cells were found to be poorly viable compared with untreated non-adherent cells (Fig. 2D). The important consequence of this reduced viability was that non-adherent cells did
not persist and there was an accumulation of cell debris in the medium of CT-treated cultures. Morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and the formation of dense rounded apoptotic bodies, were clearly evident in a high percentage of non-adherent CT-treated cells when stained with the nuclear fluorescent stain, DAPI (Fig. 3A).

Analysis by agarose gel electrophoresis of the non-adherent cells following CT treatment for 72 h in the presence of low serum (0.5% FCS) demonstrated an increase in the level of intranucleosomal genomic DNA fragmentation (Fig. 3B), a key feature of apoptosis that arises from activation of endogenous endonucleases. We were not able to assess DNA fragmentation in the adherent population due to the low number of cells recovered. CT-induced apoptosis of HR12 cells cultured in the presence of low serum was also determined by analysing phosphatidylserine externalization using Annexin V-PE/7-amino actinomycin (AV/7 AAD) staining (Vermes et al. 1995).

Figure 4A and B shows that the percentage of adherent cells that fulfilled the criteria of apoptotic cells (AV+/7 AAD−) increased significantly in the CT-treated population (0.8% vs 9.1% in the total or 0.8% vs 10.2% when recalculated as a percentage of 7 AAD− cells only). A much greater percentage of AV+/7 AAD− cells were observed in the untreated non-adherent fraction (19.5% of total, 43% of 7 AAD− cells), which further increased upon CT treatment (2.2% of total, 59% of 7 AAD− cells) (Fig. 4E, F). Furthermore, of the non-adherent population that were non-viable (7-AAD+), the percentage of AV+ cells increased markedly upon treatment with CT. This implies that the majority of non-adherent cells treated with CT represent late stages of apoptosis, consistent with the preponderance of observed apoptotic bodies visualised by DAPI staining and DNA fragmentation (Fig. 3). Flow cytometric analysis was performed on pooled adherent and non-adherent propidium iodide-stained HR12 cells (Fig. 5). A preponderance of untreated cells was in the G1 phase of the cell cycle. As we have previously reported (Evdokiou et al. 1999), CT treatment resulted in a reduction of the percentage of cells in G1 (62% vs 30%) and an increase in the percentage of cells in G2/M (12% vs 21%). Importantly, there was also an increase in the percentage of cellular DNA in cell matter corresponding to hypodiploid cells compared with control cells (5.6% vs 26%). Taken together, these data are consistent with CT treatment in a low serum concentration causing cell death by apoptosis.

**Activation of caspase-3 by CT**

We investigated whether CT could activate the effector caspase, caspase-3, whose activation is associated with the induction of apoptosis by a wide range of agents (Abu-Qare & Abou-Donia 2001). Treatment of HR12 cells with CT in 10% serum caused a modest and late
(72 h) activation of caspase-3 (Fig. 6A). In contrast, by 48 h CT treatment under low serum (0.5%) conditions, there was a dramatic activation of caspase-3, which was sustained at 72 h (Fig. 6B). CT treatment of cells in serum-free conditions caused an activation of caspase-3 that was approximately twofold greater than cells in 0.5% serum (not shown), suggesting that serum-derived factors provide protection of cells from CT-induced apoptosis.

Since cell death was associated with activation of caspase-3, the effect of caspase inhibition on cell number and viability was determined using the pan caspase inhibitors, z-VAD-fmk and Boc-D-fmk. Inclusion of caspase inhibitors completely inhibited activation of caspase-3 in both adherent and non-adherent cells (not shown). However, inhibition of caspase had no effect on the induction by CT of a population of non-adherent cells and the percentage of non-adherent cells that were viable after CT treatment (Table 1). Further, caspase inhibition did not prevent the CT-induced decrease in cell numbers seen after 3 days of CT treatment (Fig. 7). These results suggest that CT treatment resulted in a caspase-independent loss of cell adherence, leading to reduced cell survival that was associated with, but not dependent on, activation of caspase-3.

Role of MAP kinase in CT-mediated cell death

We found previously that growth suppression by CT in serum-replete conditions, of cells transfected with the insert-negative form of the hCTR, was associated with a sustained activation of the Erk1/2 MAP kinase proteins. Inhibition of Erk1/2 with PD 98059 at least partially prevented growth suppression by CT (Raggatt et al. 2000). Experiments were therefore performed to test the role of Erk1/2 in the CT-induced reduction in cell viability in 0.5% serum-containing medium. It was found that PD 98059 alone had no effect on HR12 cells, but that it

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**Figure 4** Annexin V and 7 AAD staining of adherent and non-adherent HR12 cells after CT treatment: effect of inhibition of ERK1/2 with PD 98059. HR12 cells were plated in flasks in the presence of 10% FCS and were allowed to adhere. Forty-eight hours after plating, the medium was changed to 0.5% serum and cells remained untreated (control), or were treated with 10 nM sCT, 50 μM PD 98059 or a combination of CT and 50 μM PD 98059 for a further 72 h. Adherent (A–D) and non-adherent (E–H) cells were collected separately and analysed by FACS for Annexin V (x axis) and 7 AAD (y axis) staining, as described in Materials and Methods. The percentage of cells that were either Annexin V-positive and 7 AAD-negative (AV+/7 AAD−; i.e. apoptotic cells) or Annexin V-positive and 7 AAD-positive (AV+/7 AAD+; i.e. end stage apoptotic or necrotic cells) are indicated. The percentage of apoptotic cells was calculated from the equation %AV+/7 AAD−/%(AV+/7 AAD−+AV−7 AAD−). The experiment was repeated and similar results were obtained.
partially protected cells from CT treatment in terms of the number of adherent cells and the appearance of non-adherent cells (Fig. 2A and B). Although the magnitude of the PD 98059 effect varied between experiments, qualitatively it consistently reduced the effect of CT on cell number. PD 98059 alone had no effect on the viability of adherent or non-adherent cells, as assessed by Trypan Blue dye exclusion (Fig. 2), although it did result in increased cell viability of CT-treated cells, as shown in Fig. 2D. The more sensitive Annexin V staining showed that PD 98059 was able to reduce the percentage of early apoptotic cells calculated as the percentage of 7AAD− cells that were

![Figure 5](image-url)

**Figure 5** FACS analysis of propidium iodide-stained HR12 cells after CT treatment. HR12 cells were plated in flasks in the presence of 10% FCS and were allowed to adhere. Forty-eight hours after plating, the medium was changed to 0.5% serum and cells remained untreated or were treated with 10 nM sCT, as indicated, for a further 72 h. Adherent and non-adherent cells were pooled and cell cycle analysis was performed by FACS, as described in Materials and Methods. The percentages of nuclei in the G1, S and G2/M phases of the cell cycle are indicated, as is the percentage of hypodiploid nuclei and apoptotic cell fragments (Apo). The experiment was repeated twice and similar results were obtained.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of caspase inhibitors, z-VAD-fmk and Boc-D-fmk, on the number and viability of non-adherent HR12 cells after CT treatment. Results are approximations because of the presence of lysing cells and cell debris, and are representative of two independent experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>Cell number</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
</tr>
<tr>
<td>sCT</td>
<td>10.8 × 10⁴</td>
</tr>
<tr>
<td>z-VAD-fmk</td>
<td>—</td>
</tr>
<tr>
<td>Boc-D-fmk</td>
<td>—</td>
</tr>
<tr>
<td>sCT+z-VAD-fmk</td>
<td>19.4 × 10⁴</td>
</tr>
<tr>
<td>sCT+Boc-D-fmk</td>
<td>16.2 × 10⁴</td>
</tr>
</tbody>
</table>

HR12 cells were plated at 2 × 10⁴ cells/well in 12-well plates in the presence of 10% FCS and were allowed to adhere. Seventy-two hours after plating, media were removed and replaced with 0.5% FCS-containing media. Cells were left untreated or were treated with 10 nM sCT, 50 μM z-VAD-fmk, 50 μM Boc-D-fmk, sCT+50 μM z-VAD-fmk, or sCT+50 μM Boc-D-fmk. At day 3 and day 5 following the commencement of treatment, non-adherent cells were collected by pooling cells from triplicate wells and stained with Trypan Blue for quantitation of viable and non-viable cells. The corresponding total cell numbers and caspase-3 activity of adherent and non-adherent cells is shown in Fig. 4.
AV in both the adherent (10·2% vs 4·7%, Fig. 4B, D) and non-adherent (59% vs 41%, Fig. 4 F, H) cell populations. Consistent with the reduced effects of CT on cell number and cell viability, PD 98059 also reduced the activation of caspase-3 in CT-treated cells (Fig. 8).

Discussion

These data indicate that CT can influence the survival of cells expressing the insert-negative isoform of the human CTR. The CTR is a member of the 7 membrane-spanning, G protein-coupled receptor subfamily that includes the receptors for parathyroid hormone (PTH), secretin, pituitary adenylate cyclase-activating polypeptide (PACAP) and glucagon, which was designated Family B by Kolakowski (1994). There are now many reports of members of this receptor family participating in cell survival decisions (reviewed in Sherwood et al. 2000), which are of likely significance in tissue morphogenesis and repair. While many of these reports suggest a protective role against apoptosis for these receptor systems (Delgado et al. 1996, Villalba et al. 1997), it is clear that pro-apoptotic effects are also important. Enhancement of apoptosis has been reported for calcitonin–gene-related peptide (CGRP; Sakuta et al. 1996, Millet et al. 2000), and for PTH/PTH-related protein (PTHRP) (Turner et al. 2000, Divieti et al. 2001) in a number of cell types. In the case of the CT/CTR system it is evident that diverse functions exist, including morphogenesis, based on the widespread tissue localisation of both the receptor and the ligand (Sexton et al. 1999), their expression from early embryological development (Wang et al. 1998, Zhu et al. 1998), and the clear ability of CT to affect cell survival.

Activation of caspase-3 was associated closely with the decreased number of adherent cells and an increase in number of non-viable non-adherent cells. However, the results are consistent with cell death occurring secondary to a loss of cell attachment, and independent of caspase activation, since both loss of attachment and cell death proceeded in the presence of caspase inhibition. Thus, activation of caspase-3 by CT was a consequence, rather than a cause, of decreased cell viability in the present experiments. With respect to the redundancy of caspase activation in CT-mediated cell death, several recent reports have drawn attention to modes of programmed cell death that are both caspase-dependent and caspase-independent (e.g. Fehlberg et al. 2002, reviewed by Borner & Monney 1999).

In general, the viability of adherent cells is dependent on attachment, and cell death by a process termed anoikis is induced by loss of adequate and appropriate cell–matrix interactions. It is apparent that CT can mediate divergent effects on cell attachment, depending on cell type and cellular circumstances. In osteoclasts, CT was shown to protect adherent cells, but not floating cells, from anoikis (Sakai et al. 2000). In contrast, our results show that CT promotes anoikis in HEK-293 cells in low-serum conditions, but not in serum-replete conditions. It is well known that certain growth factors, of which serum is a rich source, can promote cell survival as well as positively affect cell proliferation. It appears that a deficiency of these factors in low-serum conditions renders HEK-293 cells over-expressing the CTR, which are strongly growth inhibited by CT in serum-replete conditions (Evdokiou et al. 1999), susceptible to CT-induced anoikis. As stated, the initiator of cell death in serum-deplete conditions was probably loss of proper attachment, which was observable in the rounding up of cells after CT treatment. Integrins play a vital role in cell adherence, and three primary integrin signalling molecules that have been linked to cell survival are focal adhesion kinase (FAK), Shc and integrin-linked kinase (ILK) (reviewed by Frisch & Screaton 2001). Two recent reports provide a link between CT treatment of HEK-293 cells and integrin-mediated events and enable a partial explanation for the ability of CT to affect cell attachment. These reports show that CT influenced focal adhesion and cytoskeletal components in HEK-293 cells over-expressing the C1a isoform of the rabbit CTR (Zhang et al. 1999, 2000). CT was shown to induce phosphorylation of the focal adhesion-associated protein, human enhancer of filamentum 1 (HEF 1), paxillin and FAK, as well as the association of these latter two proteins with HEF 1. This effect of CT required cell attachment and the integrity of the cytoskeleton, and involved c-Src. These results show that CT treatment can result in complex effects on cell attachment mechanisms, although it is not yet clear how these results relate to the effect of CT cell survival, measured over days, since the reported experiments were short term (up to 60 min).

We found previously that growth suppression by CT was associated with a sustained activation of the p42/44 MAP kinase proteins (Raggatt et al. 2000). Inhibition of p42/44 MAP kinase with PD 98059 at least partially prevented growth suppression by CT. The evidence reported here indicates that this pathway was also important in CT-induced loss of cell viability, since inhibition of Erk1/2 reduced the induction of cell death by CT in HR12 cells. The role of the Erk1/2 pathway in CT-mediated effects on cell attachment is likely to be complex, with interactions between other intracellular signalling pathways and substrate-dependent actions of this pathway either leading to or protecting from anoikis (Gu et al. 2002). However, two distinct types of interactions between Raf-MEK-Erk and integrin signal transduction have been identified. The first of these, known as ‘outside-in’ signalling, involves the promotion of various signalling pathways, including the Raf-MEK-Erk pathway, by integrin engagement (Short et al. 2000). The second mode
of integrin regulation, known as ‘inside-out’ signalling, is where the Erk pathway can influence the activation state of integrins. In one report, suppression of integrin activation correlated with activation of the Erk-MAP kinase pathway (Hughes et al. 1997). More recently, sustained activation of the Raf-MEK-Erk pathway was shown to lead to decreased focal adhesions and actin stress fibres, and a decreased spreading and adhesion of NIH 3T3 cells, despite a paradoxical increase in β3-integrin mRNA and an induction of α5β3 integrin expression on the cell surface (Woods et al. 2001). These reports suggest future areas of investigation with respect to the effects of CT on cell attachment machinery, but draw attention to the multiple levels of regulation of integrin attachment that will need to be explored in this context.

The results described here point to a novel action of CT to regulate cell survival, perhaps by effects on components of the cell attachment mechanism. The work has implications for understanding the actions of CT in the inhibition of osteoclast activity and also in the potential use of CT to control cancer cell growth, given the frequent alteration of p53 gene. Experimental Neurology 167 183–188.


Zhang Z, Baron R & Horne WC 2000 Integrin engagement, the actin cytoskeleton, and c-Src are required for the calcitonin-induced tyrosine phosphorylation of paxillin and HEF1, but not for calcitonin-induced Erk1/2 phosphorylation. *Journal of Biological Chemistry* **275** 37219–37223.


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