Cell Death and Differentiation (2000) 7, 189–196

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Cripto-1 induces apoptosis in HC-11 mouse mammary epithelial cells

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Received 15.4.99; revised 11.8.99; accepted 23.8.99
Edited by C Thiele

Abstract

Cripto-1 (CR-1) is an epidermal growth factor (EGF)-related protein. CR-1 can inhibit β-casein and whey acidic protein expression in mouse mammary epithelial cells. The present study demonstrates that CR-1 can induce apoptosis in HC-11 mouse mammary epithelial cells, as measured by bis-benzimide stained nuclei, TUNEL assay and cell death ELISA. Apoptosis could be observed after 2 days of exposure of confluent HC-11 cells to CR-1 in the absence of the survival factors EGF and insulin, with maximum apoptosis occurring at 3 days. A reduction in poly(ADP-ribose) polymerase (PARP) expression and an increase in β-catenin cleavage was found after 18 h of exposure to CR-1 suggesting that apoptosis was preceded by the induction of a caspase activity since the caspase inhibitor ZFAD.FMK could block the CR-1-induced reduction in PARP expression and CR-1-induced apoptosis. CR-1 was found to increase the expression of caspase-3-like protease. Although, the levels of $p27^{kip1}$ and p21Bax did not change after exposure to CR-1 for 18 h, the levels of Bcl-x_L became undetectable. These studies suggest that CR-1 promotes apoptosis by mediating the induction of caspase-3-like protease and downregulating the expression of Bcl-x_L.
Cell Death and Differentiation (2000) **7,** 189–196.

Keywords: apoptosis; Cripto-1; caspase activation; mammary epithelial cells

Abbreviations: CR-1, cripto-1; EGF, epidermal growth factor; PARP, poly(ADP-ribose) polymerase; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethyl ketone; BOC, Boc-Asp(Ome) fluoromethyl ketone

Introduction

During normal mammary gland development, programmed cell death (PCD) and cell proliferation play a major role in controlling tissue remodeling and growth.[1] Processes such as ductal morphogenesis, lobulo-alveolar development during pregnancy and lactation and involution are characterized by different degrees of apoptosis and proliferation.[2,3] These biological mechanisms are controlled by an interplay of hormones, growth factors, cell-cell and cell-matrix interactions.[4] One of the locally-derived growth factor families that has been implicated in these processes is the epidermal growth factor (EGF) family.[1,5] Both EGF and transforming growth factor-α (TGF-α) can stimulate the proliferation of mammary epithelial cells and lobulo-alveolar development in the mammary gland of virgin mice.[1,6] These growth factors are also able to modulate milk protein expression in mammary epithelial cells when cells are exposed to lactogenic hormones[7] and can inhibit apoptosis in secretory alveolar epithelial cells in the involuting mammary gland when ectopically overexpressed.[8]

We recently identified another EGF-related protein, CR-1 (cripto-1) that can also modulate mammary epithelial cell morphogenesis and differentiation.[7,9–12] CR-1 is a member of a larger family of structurally related proteins, the EGF-CFC family that also includes, mouse Cr-1, mouse cryptic, *Xenopus* FRL-1 and zebrafish *oep*.[7] These proteins perform an essential function during early vertebrate embryogenesis by promoting the formation of mesoderm during gastrulation and by facilitating the initial migration of epiblast cells from the primitive streak. Human CR-1 is a 28-kD glycoprotein cotaining 188 amino acids. CR-1 contains three intra-disulfide bonds encompassing a modified EGF-like consensus sequence of approximately 37 amino acids. However, unlike other EGF-like proteins, the human CR-1 protein lacks a conventional hydrophobic signal peptide and transmembrane domain and the modified EGF-like motif which is also possessed by other members of the EGF-CFC family, lacks the A loop, while the B loop is truncated.[13] Another feature that distinguishes CR-1 from other EGF-like peptides is the failure of CR-1 to directly bind to any of the known c-*erb*-B type 1 receptor tyrosine kinases.[13] However, CR-1 can indirectly induce a transient increase in the tyrosine transphosphorylation of c-*erb*-B 4 in mouse and human mammary epithelial cells.[14] CR-1 can inhibit β-casein and whey acidic protein (WAP) expression *in vitro* in mouse mammary epithelial cells.[9,11,12] CR-1 can also induce *in vitro* and *in vivo* branching morphogenesis in mouse mammary epithelial cells.[7,10,11] Cr-1 is normally expressed at a low level in a subpopulation of mammary epithelial cells within the growing terminal end buds of the virgin mouse mammary gland.[15,16] However, Cr-1 expression in ductal epithelial cells increases during pregnancy, lactation and also in the aged mouse mammary

gland.[10,12,15,16] CR-1 mRNA and immunoreactive CR-1 protein are expressed in several human breast cancer cell lines, in approximately 80% of primary human breast carcinomas and in several different types of spontaneous and transgenic mouse mammary tumors.[17–20] With respect to intracellular signaling, CR-1 is able to activate the ras/raf/MEK/MAPK and the PI3-kinase signaling pathways.[9,11,13] In this regard, the activation of $p21^{ras}$ and PI3-kinase has been linked to the ability of CR-1 to inhibit the expression of β-casein and WAP in mouse mammary HC11 epithelial cells and in primary cultures of midpregnant mouse mammary epithelial cells.[9]

The present study was designed to ascertain if CR-1 might modulate apoptosis in HC-11 mouse mammary epithelial cells under conditions where β-casein expression is inhibited. CR-1 was found to induce apoptosis in serum-restricted confluent cultures of HC-11 cells through a caspase-dependent process since several proteins that are substrates for caspases such as poly(ADP-ribose) polymerase (PARP) and β-catenin[21–23] were found to degraded after CR-1 treatment. In addition, we have found that CR-1 decreases the expression of bcl-x_L but not p21 bax or $p27^{kip1}$ expression.[24,25]

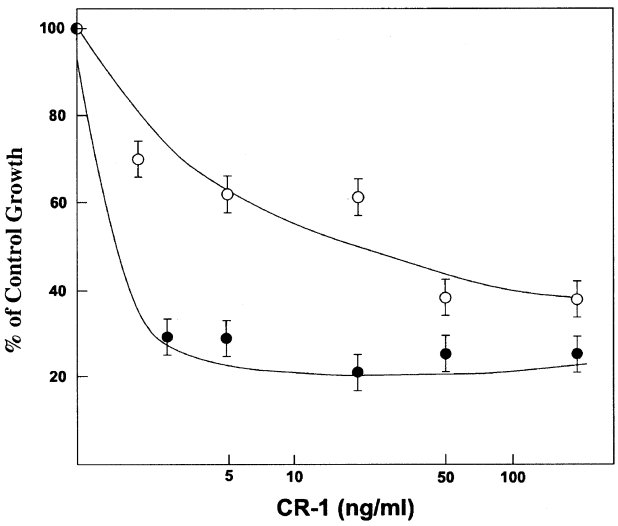

Figure 1 CR-1 inhibits mammary epithelial cell growth. HC-11 cells were seeded at 2×10^3 cells/well in a 96 well-tissue culture plate in RPMI 1640 medium containing 3% Fetal Bovine Serum (FBS). The cultures were treated with increasing concentrations of CR-1 for 48 (○) and 72 h (●). The cells were incubated with tetrazolium salt (WST-1) for 4 h and the formed formazan dye was quantitated using an ELISA reader

Results

CR-1 inhibits HC-11 mammary epithelial cell growth under serum-restricted conditions

Compared to EGF, CR-1 is a relatively weak mitogen *in vitro* for human and mouse mammary epithelial cells.[9,12,26] To ascertain if CR-1 might modulate mammary epithelial cell apoptosis, we used HC-11 mouse mammary epithelial cells, a clone derived from the mouse mammary epithelial COMMA-D cell line.[27] It has previously been reported that confluent HC-11 cells undergo apoptosis under serum-free medium conditions in the absence of the survival factors, EGF or insulin.[28] Therefore, proliferation of HC-11 cells in sparse cultures in response to CR-1 was assessed in 3% FBS containing medium (Figure 1). After 48 h or 72 h of exposure to different concentrations of CR-1, HC-11 cell growth was reduced by nearly 70% after 72 h with 2.5 ng/ml as compared to control cultures (no CR-1 added) (Figure 1).

CR-1 promotes apoptosis in HC-11 mammary epithelial cells

To ascertain if the growth inhibitory effects of CR-1 in low serum might be associated with any effect CR-1 might have on apoptosis, confluent HC-11 cells that were depleted of EGF or insulin, were exposed for either 1–5 days to CR-1 (100 ng/ml) in low serum and in the absence of EGF or insulin. These conditions were selected since there is an increase in the response of HC-11 cells to apoptosis at confluency as compared to sparse subconfluent cells with respect to growth factor withdrawal.[42] After 2 days of exposure to CR-1 (100 ng/ml), a significant number of confluent HC-11 cells exhibited detectable signs of apoptosis, namely, nuclear fragmentation and the appearance of apoptotic bodies (Figure 2, Table 1). These effects were maximum after a 3 day exposure.

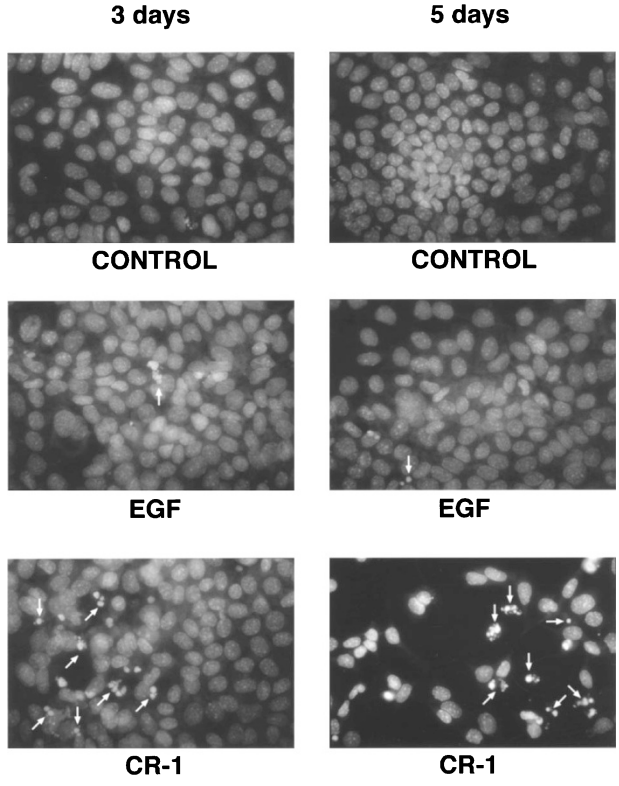

Figure 2 CR-1 induces apoptosis in HC-11 cells. HC-11 cells were grown to confluency and then maintained in 3% FBS containing medium with or without CR-1 (100 ng/ml) or EGF (100 ng/ml) for 3 and 5 days. Cell nuclei were stained with *bis*benzimide (Hoechst 33258) and apoptosis was assessed by nuclear morphology. Evidence of apoptosis is indicated by the white arrows, i.e., contraction of the cell body, condensation of nuclear chromatin and fragmentation, and the production of apoptotic bodies

Approximately 17–19% of the HC-11 cells were apoptotic in CR-1-treated HC-11 cells as ascertained by bis-benzimide staining in the majority of the fields that were analyzed (Figure 2, Table 1). These findings were confirmed using two additional apoptosis assays on confluent HC-11 cells, the TUNEL assay (Figure 3) and the cell death ELISA (Figure 5).

Table 1 Quantification of apoptosis mediated by CR-1

	% apoptotic cells	
Days in the presence of CR-1	**Control**	**CR-1**
1	1.34	0.9
2	0.65	13.9
3	1.3	16.6
4	2.1	16.7
5	2.4	19.8

Cell nuclei were stained with *bis*benzimide (Hoechst 33258) and apoptosis was assessed by nuclear morphology. CR-1 was used at 100 ng/ml. Three random fields per exposure were analyzed. The number of experiments performed independently were two per each condition

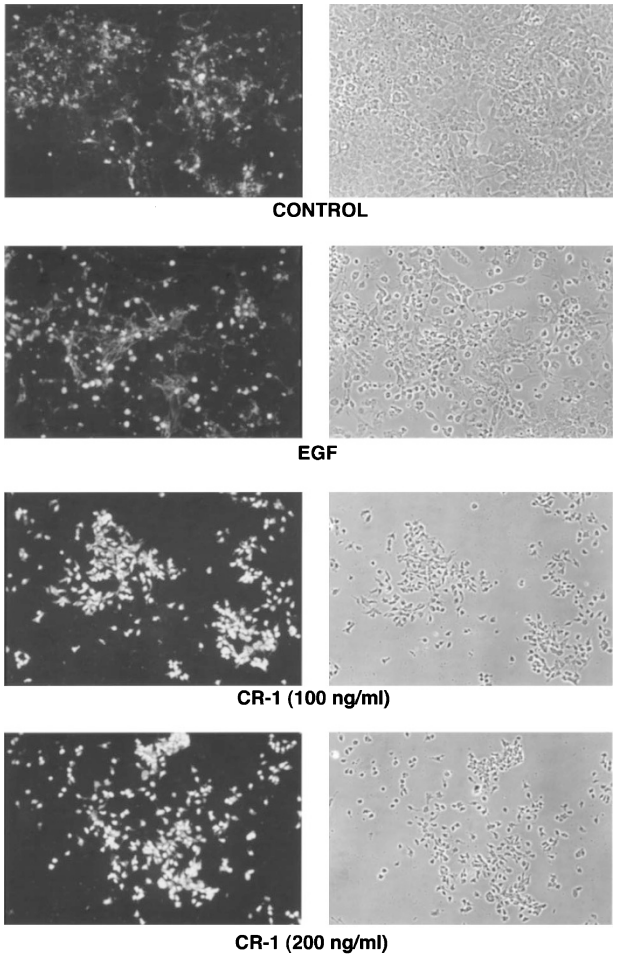

Figure 3 CR-1 induced-apoptosis as assessed by TUNEL assay. HC-11 mammary epithelial cells were grown to confluency and then maintained in 3% FBS containing medium with or without CR-1 (100 or 200 ng/ml) or EGF (100 ng/ml) for 5 days

Figure 3 shows that the majority of CR-1 treated HC-11 cells were positive in the TUNEL assay. After 5 days of exposure, the amount of HC-11 cells undergoing apoptosis in response to CR-1 was dramatically higher when compared to the control cell cultures (Table 1). In addition, CR-1 was able to promote apoptosis as assessed by Hoechst staining in HC-11 cells in a dose-dependent manner (Figure 4). After 5 days of exposure to CR-1, HC-11 cells showed significant apoptotic features even when they were exposed to 2.5 ng/ml.

CR-1 induces caspase-mediated apoptosis in HC-11 mammary epithelial cells

To further define the biochemical processes that might be involved in the CR-1-mediated apoptotic effect on HC-11 cells, caspase activation was studied since caspase induction and / or activation is an important feature of the apoptotic signaling pathway that normally precedes nuclear fragmentation.[22] By using a cell death ELISA, DNA fragmentation and therefore an index of apoptosis could be quantified in confluent HC-11 cells that had been exposed to 100 ng/ml of CR-1 in the absence of EGF or insulin (Figure 5). In order to establish the involvement of caspases in this process, a general caspase inhibitor BOC (10 μM) was used. As shown in Figure 5, the caspase inhibitor was able to completely abolish CR-1-mediated apoptosis. A comparable inhibitory effect was also observed when 20 μM ZVAD.FMK caspase inhibitor was used, suggesting that CR-1 may activate the caspase-3 subfamily of proteases and then induce apoptosis.[29]

CR-1-mediated caspase activation is associated with decreased PARP expression and β-catenin cleavage in HC-11 cells

Poly(ADP-ribose) polymerase (PARP) is one of the main substrates for caspases.[30] Therefore, the steady state levels

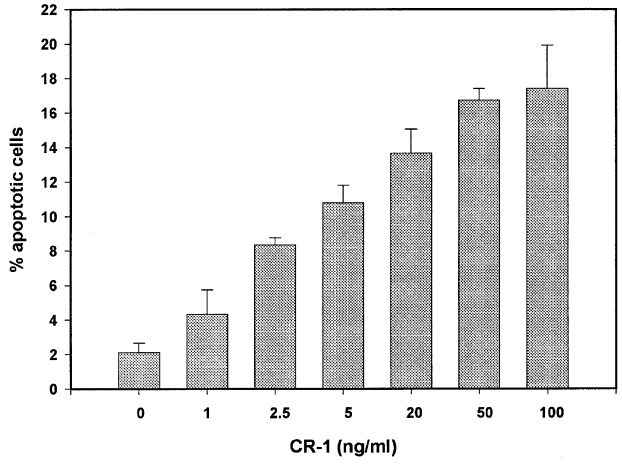

Figure 4 CR-1 induces apoptosis in HC-11 cells in a dose-dependent manner. Apoptosis was assessed by nuclear morphology using *bis*benzimide (Hoechst 33258) staining in confluent HC-11 cells mantained in 3% FBS containing medium after 5 days of CR-1 exposure. Results are expressed as the mean $\pm$ S.E.

of intact PARP protein expression were assessed by Western blot analysis in lysates from confluent HC-11 cells that had been exposed to CR-1. CR-1 (100 ng/ml) was able to induce a significant reduction in the levels of intact p116 PARP protein following an 18 h treatment (Figure 6). No evidence of a decrease in PARP levels was found at 10 h or 12 h after exposure to CR-1 (data not shown). The reduction in PARP levels in response to CR-1 was partially blocked with the general caspase inhibitor BOC and completely with 20 μM of the caspase-3 inhibitor ZVAD.FMK, suggesting that the reduction in PARP levels might be due to the activation of a caspase-3-like protease. When 20 μM of the inactive caspase inhibitor, ZAFAFK.FMK was added to HC-11 cells, CR-1 was still able to induce a reduction in PARP levels (Figure 6).

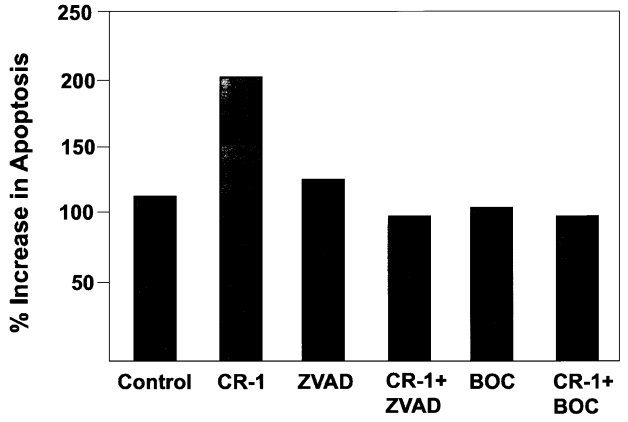

Figure 5 Inhibition of CR-1-mediated apoptosis by caspases inhibitors. Apoptosis was quantitated by detecting apoptotic DNA fragments in lysates using histone-DNA ELISA. HC-11 cells were grown until confluency and exposed to CR-1 (100 ng/ml) with or without inhibitors for 36 h. ZVAD.FMK caspase inhibitor (ZVAD) was used at 20 μM and the general caspase inhibitor BMD.FMK (BOC) was used at 10 μM. The cells were preincubated for 1 h with the caspase inhibitors prior to the addition of the growth factors. The results are expressed as the mean of two separate experiments

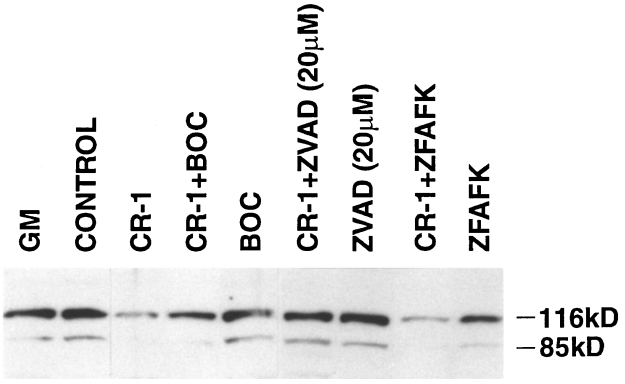

Figure 6 CR-1 reduces PARP expression. HC-11 cells were grown until confluency and treated with CR-1 (100 ng/ml) with or without caspase inhibitors for 18 h. The cells were preincubated for 1 h with the caspase inhibitors prior to the addition of CR-1. ZVAD.FMK caspase inhibitor (ZVAD) was used at 20 μM, BMD.FMK general caspase inhibitor (BOC) was used at 10 μM and ZFAFK.FMK, caspase inhibitor negative control, was used at 20 μM. The results of one experiment which is representative of three, are shown. PARP protein expression was determined by Western blot analysis of treated HC-11 cell lysates using anti-PARP antibodies. The intact full length PARP protein (116-kD) and the 85-kD fragment are denoted by an arrow respectively

To more fully define the involvement of caspase-3-like activation in CR-1-mediated apoptosis, the levels of β-catenin, a caspase-3 substrate, were analyzed in HC-11 cells.[21] As shown in Figure 7, CR-1 at either 10 or 100 ng/ml induced the cleavage of the 92 kD β-catenin protein to a $\sim$90 kD fragment in confluent HC-11 cells after an 18 h incubation period. The appearance of this intermediate $\sim$90 kD β-catenin degradation fragment has been previously reported.[21] In contrast to β-catenin, the levels of α-catenin were not significantly altered in confluent HC-11 cells after exposure to CR-1. In order to correlate this caspase activity with the presence of the caspase-3 precursor protein, CPP32, the levels of CPP32 protein expression were measured in HC-11 cells by Western blot analysis. The levels of CPP32 protein were extremely low in control untreated HC-11 cells or in cells treated with 10 ng/ml of CR-1. In contrast, a threefold increase in CPP32 expression was observed in HC-11 cells that had been treated with CR-1 (Figure 7).

Effect of CR-1 on the expression of apoptotic associated proteins in HC-11 cells

To ascertain if CR-1 might affect the expression of several key pro- or anti- apoptotic proteins, the levels of bcl-x_L, $p27^{kip1}$ and p21 bax were studied.[31,32] The anti-apoptotic protein, bcl-x_L, was found to be expressed in confluent HC-11 mammary epithelial cells (Figure 8). However no bcl-x_L expression was found in confluent HC-11 after exposure to CR-1 (Figure 8). The levels of $p27^{kip1}$, a negative regulator of the cell cycle, did not change in response to CR-1 exposure. With respect to the pro-apoptotic protein p21 bax, the levels of this protein remained unchanged after CR-1 treatment of HC-11 cells (Figure 8).

Discussion

Growth and differentiation in the mammary gland are regulated by a coordinated interplay of systemic mammotrophic hormones and locally-derived growth factors.[1,33] The complexity of the biological roles of these factors is not completely understood. In an attempt to more fully elucidate the potential role that a novel growth factor and morphogen CR-1 might perform in mammary epithelial cells, we have studied the effects of CR-1 on the differentiation of a normal mammary epithelial cell line, HC-11. HC-11 cells resemble midpregnant mammary epithelial cells in that they express the milk protein, β-casein in response to lactogenic hormone stimulation.[28] CR-1 can inhibit the expression of the milk protein, β-casein, in HC-11 cells when these cells are simultaneously exposed to lactogenic hormones at confluency.[9] This may be physiologically significant *in vivo* since elevated expression of mouse Cr-1 occurs in the pregnant and lactating mouse mammary gland.[12,15] In this report we have identified another role of CR-1, namely, its ability to promote apoptosis in confluent, survival-factor-depleted HC-

11 mammary epithelial cells. This effect is preceded by the activation of a caspase-3-like protease(s). This conclusion is based on several observations. First, the ZVAD.FMK caspase inhibitor can block CR-1-induced apoptosis in HC-11 cells

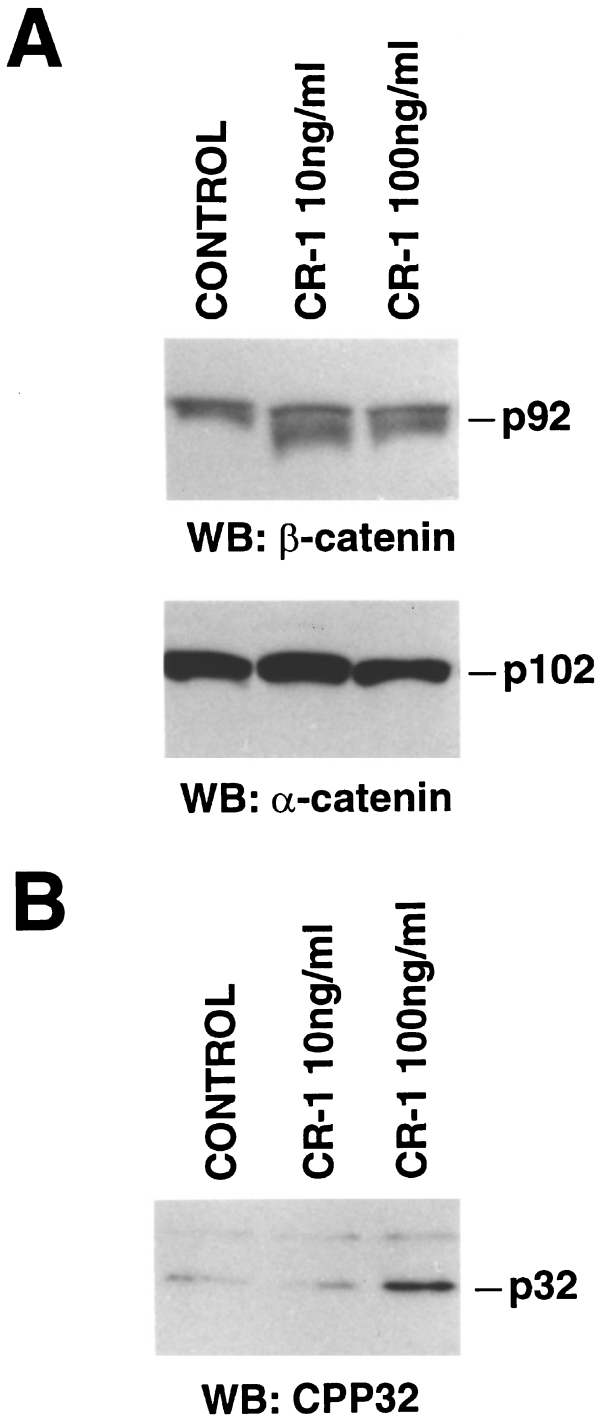

Figure 7 β-catenin, α-catenin and CPP32 protein expression in HC-11 cells exposed to CR-1 (100 ng/ml) in RPMI 1640 medium containing 3% FBS for 18 h. Western blot analyses using anti- β catenin, anti- α-catenin or anti-CPP32 antibodies were performed as described in Materials and Methods

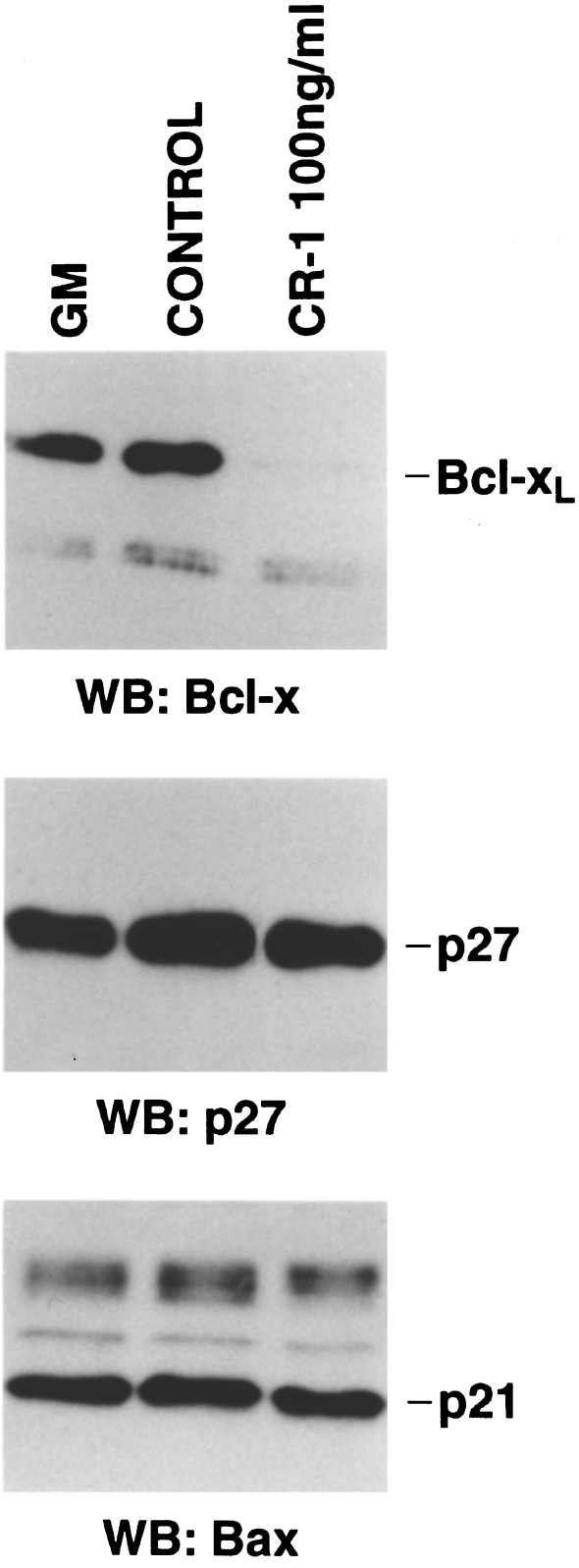

Figure 8 Bcl-x, $p27^{kip1}$ and p21 Bax protein expression in mammary epithelial HC-11 cells treated with CR-1 (100 ng/ml) in RPMI 1640 medium containing 3% FBS; or GM (10% FBS containing medium) for 18 h. Western blot analyses using anti-Bcl-x, anti-$p27^{kip1}$ and anti-p21 Bax antibodies were performed as described in Materials and Methods

when used at concentrations that are sufficient to inhibit caspase-3 activation.[29]

Second, substrates for caspase-3 like proteases such as PARP and β-catenin were found to be either reduced or cleaved in CR-1 treated HC-11 mammary epithelial cells, respectively. Finally, CR-1 downregulated the expression of the anti-apoptotic protein, bcl-x_L, without affecting the expression of p21 bax. This effect is distinct from the survival promoting effects of EGF which upregulate the expression of bcl-x_L in mammary epithelial cells.[50] These findings support the possibility that CR-1 may be activating a caspase-3-like protease, since it has been reported that bcl-x_L can be cleaved *in vitro* by activated caspase-3.[34] Human mammary epithelial cells generally show low or undetectable levels of CPP32, the precursor protein of caspase-3 as compared to human breast cancer cells.[35] In accord with this observation, the level of CPP32 protein in HC-11 mouse mammary epithelial cells was nearly undetectable. CR-1 upregulated CPP32 expression in confluent growth-factor deprived HC-11 cells. This effect might contribute to the apoptotic process observed in confluent HC-11 cell cultures. Since β-catenin is found in the adherens junctions of epithelial cells with E-cadherin and is involved in regulating homotypic cell-cell adhesion, then degradation of β-catenin would potentially lead to a loss of cell contact.[21,36] This effect may be important not only with respect to the initiation of apoptosis but also in regulating the ability of CR-1 to stimulate branching morphogenesis.[10,11,21] Analogously, it has been recently shown that hepatocyte growth factor, which also induces branching morphogenesis of mouse and human mammary epithelial cells, can promote apoptosis by increasing caspase-3 activity.[37]

Another important factor to be considered is that CR-1 is facilitating apoptosis in confluent cultures of HC-11 cells that have been depleted of two crucial survival factors, EGF and insulin.[38] This correlates with a previous report demonstrating that HC-11 cells in high density cultures have a reduced contact with a solid substrate, which is known to facilitate the onset of apoptosis through a process known as anoikis.[38,39] Under these culture conditions, the levels of the p21 bax protein increase in mammary epithelial cells.[38,40,41] High levels of p21 bax protein are normally observed in confluent HC-11 cells.[38] However, high cell density and therefore, high levels of p21 bax expression are not entirely sufficient to induce apoptosis.[38] Addition of pro-apoptotic cytokines such as CR-1 may be equally important at an early stage in this system in triggering apoptosis. Since HC-11 cells lack wild type p53,[42] CR-1 is probably inducing apoptosis in this system through a p53-independent pathway. In contrast to these results, Niemeyer *et al* recently found that in CID-9 mouse mammary epithelial cells that were transfected with a mouse Cr-1 expression vector, growth was modestly stimulated and apoptosis under serum-restricted conditions was reduced by twofold.[12] However, since CID-9 cells were assessed for apoptosis under sparse culture conditions in medium containing serum and insulin and since no indication was presented as to the amount of mouse Cr-1 protein that was secreted by these cells, it is difficult to compare this data with the results obtained on confluent HC-11 cells that have been treated with purified recombinant human CR-1 in the absence of EGF or insulin. In addition, in HC-11 cells, apoptosis is more pronounced in serum-restricted confluent cultures than in sparse or subconfluent cultures.[38]

Finally, CR-1 expression is elevated in a majority of mouse and human mammary tumors relative to noninvolved mammary epithelium.[7,17,18,20] In breast hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive carcinomas, the degree of apoptosis has been shown to be correlated with increased malignancy.[3,43] It is therefore conceivable that CR-1 may be one factor that is contributing to the elevated apoptotic index in these lesions since CR-1 expression can first be detected in hyperplasias in different transgenic mice that eventually develop mammary tumors and in human DCIS.[5,10,20,51] Overexpression of Cr-1 in NOG-8 or CID-9 mouse mammary epithelial cells *in vitro* leads to transformation *in vitro* as evidenced by an enhanced anchorage-dependent growth in the absence or presence of serum, a loss of contact inhibition of growth and an increase in anchorage-independent cell growth.[10,12,44] However, both NOG-8 and COMMA-1D mouse mammary epithelial cells that were overexpressing either human CR-1 or mouse Cr-1, respectively, were unable to form tumors in either nude mice or Balb/c mice, respectively.[12,44] The finding that CR-1 is involved in both inducing apoptosis and promoting the early stages of transformation is analogous to the effects that are produced by another EGF-related growth factor, heregulin. Heregulin is primarily expressed in the stroma of the midpregnant mouse mammary gland and has also been shown to induce apoptosis in mouse and human mammary epithelial cells and to enhance the early stages of tumor formation in the mouse mammary gland.[45–49] In conclusion, cell proliferation and apoptosis in mammary epithelial cells reflect a complex balance between two processes that are controlled by systemic mammotrophic and lactogenic hormones and locally-derived growth factors. A better understanding of these processes will help to elucidate the significant role that growth factors play in regulating normal mammary gland physiology and neoplasia.

Materials and Methods

Cell culture

HC-11 mouse mammary epithelial cells were grown until confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 ng/ml EGF, and 5 μg/ml insulin (GM). Confluent HC-11 cells were kept in GM medium for 48 h and then treated with CR-1 in RPMI 1640 medium containing 3% FBS. Treatment of HC-11 cells with 20 μM caspase inhibitor, ZVAD.FMK (ZVAD); 20 μM of the general caspase inhibitor BMD.FMK (BOC) or with 20 μM negative control, ZFAFK.FMK, was initiated 1 h prior to the addition of CR-1. All caspase and control inhibitors were purchased from Enzyme Systems Products; Dublin, CA, USA. No cellular toxicity was observed with the concentrations of inhibitors that were used in the experiments.

Cell proliferation was assessed in non-confluent HC-11 cell cultures using WST-1 reagent (Boehringer-Mannheim, Indianapolis, IN, USA). Briefly, HC-11 cells were plated in 96-well plates at 2×10^3

cells per well in 3% FBS containing medium. The cells were incubated with CR-1 for 48 and 72 h. WST-1 was added during the last 4 h of the incubation period as recommended by the manufacturer. The color developed was quantified using an ELISA reader and read at 450 nm.

Detection of apoptotic cells

Bis-benzimide staining Morphological changes in the nuclear condensation of chromatin of cells undergoing apoptosis were assessed by staining with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258; Sigma) as described elsewhere.[50] In brief, HC-11 cells were plated in polysterene chamber slides (LabTek) at 5×10^4 until confluent. The cells were then kept for 48 h in GM medium and then treated with or without CR-1 at different concentrations in the presence of 3% FBS for different times. After treatment, the cells were washed twice with PBS and incubated with 3.5% paraformaldehyde in PBS for 20 min at room temperature. After fixation, the cells were washed twice with PBS and stained with bis-benzimide (16 μg/ml) in PBS. Following 15 min of incubation at room temperature, the cells were washed twice with PBS and the average number of nuclei per field was scored for the incidence of apoptotic chromatin under a fluorescence microscope. Cells with three or more condensed chromatin fragments were considered apoptotic.

TUNEL assay HC-11 cells were plated in polysterene chamber slides (LabTek) at 5×10^4 until confluence. The cells were kept in GM medium for 48 h and then treated with or without CR-1 (100 or 200 ng/ml) or EGF (100 ng/ml) in the presence of 3% FBS for different times. After treatment, the cells were washed twice with PBS and incubated with 3.5% paraformaldehyde in PBS for 20 min at room temperature. DNA fragmentation in apoptotic cells was determined by measuring terminal deoxynucleotidyltransferase activity by the terminal de-oxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, (TUNEL) assay (Boehringer-Mannheim, Indianapolis, IN, USA). TUNEL-positive cells were examined using a fluorescence microscope.

DNA fragmentation cell death ELISA HC-11 cells were plated in 48-well plates at 1×10^4 cells per well and grown until confluence. The cells were kept in GM medium for 48 h and then treated with or without CR-1 (100 ng/ml) in the presence or absence of caspase inhibitors at the indicated concentrations in RPMI 1640 medium containing 3% FBS for 36 h. After incubation, cytoplasmic nucleosomal DNA fragments were assessed using an apoptotic cell death ELISA (Boehringer Mannheim) with anti-histone and anti-DNA antibodies, as previously described to assess DNA fragmentation.[50] The color developed was quantified using an ELISA reader and read at 405 nm.

Western blot analysis

Cells were lysed and homogeneized in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM $MgCl_2$, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 20 mM sodium fluoride. After clarification, 100 μg/sample of the protein lysates were separated by SDS–PAGE, transferred to nitrocellulose and blocked with 4% dry milk in 20 mM Tris-buffered saline with 0.05% Tween 20. The blots were incubated with either a 1 : 1000 dilution of a rabbit anti-PARP antibody (Biomol, Pennsylvania, USA), a 1 : 1000 dilution of both mouse monoclonal anti-β-catenin and monoclonal anti-α-catenin antibodies from Transduction Laboratories (Kentucky, USA); a 1 : 400 dilution of a rabbit anti-CPP32 antibody (Upstate Biotechnology Inc., New York, USA); or a 1 :1000 dilution of either rabbit anti-$p27^{kip1}$, rabbit anti-bcl-x or rabbit anti-bax antibodies from Santa Cruz Biotechnology Inc. (California). The bound rabbit or mouse antibodies were detected using a 1 : 3500 dilution of either goat-anti-rabbit or goat-anti-mouse IgG conjugated to horseradish peroxidase (Amersham Corp.). Quantitation of bands was measured by densitometric analysis.

Acknowledgements

AD Ebert was supported by DFG grant Eb 152/42.

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