Type V Collagen Regulates the Expression of Apoptotic and Stress Response Genes by Breast Cancer Cells

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Type V collagen is a "minor" component of normal human breast stroma, which is subjected to over-deposition in cases of ductal infiltrating carcinoma (DIC). We reported that, if used as a culture substrate for the DIC cell line 8701-BC, it exhibited poorly-adhesive properties and restrained the proliferative and motile behavior of the cell subpopulation able to attach onto it. Moreover, this collagen species was able to trigger DNA fragmentation and impair survival of 8701-BC cells. In this study, we have extended our investigation with the aim to obtain further evidence that the death induced by type V collagen was of the apoptotic type by (i) microscopic detection and quantitation of Apoptag-labeled cells, (ii) analysis of the expression levels of selected genes coding for apoptosis-linked factors, caspases, and stress-response proteins by conventional and semi-quantitative multiplex PCR, and (iii) evaluation of the extent of caspase activation by chromogenic assay. We report here that type V collagen is able to determine an increase in the percentage of Apoptag-positive cells, to up-regulate Bcl-xS, Bad, Dap kinase, hsf-1, mthsp75, caspase-1, -5, -8, -9, and -14, whilst down-regulating Bcl-2, Bcl-xβ, and hsp60. Treatment of cell lysates with chromogenic tetrapeptide substrates specific for caspase-1, -5, -8, and -9 demonstrated a marked increase of enzymatic activity in the presence of type V collagen. Our data validate 8701-BC cell line as a suitable "in vitro" model for further and more detailed studies on the molecular mechanisms of the death response induced by type V collagen on primary DIC cells. J. Cell. Physiol. 202: 411–421, 2005. © 2004 Wiley-Liss, Inc.

Apoptosis, which is one of the described patterns of programmed cell death (Leist and Jäättelä, 2001), is a process of cell elimination characterized by an ordered series of genetically-controlled events, essential for both preservation of tissue homeostasis and effective protection against viral infections and cancer (White, 1996 for a review). Although the phenotypical aspects of apoptotic steps are well-known and constant in the different organisms, the molecular bases of the intracellular cascade leading to cell death, which are complex and markedly diversified, have been only partially-elucidated. The major event associated with "classical" apoptosis is the activation of a family of intracellular cysteine proteases, i.e., the caspases, which is regulated directly or indirectly by the products of a consistent number of death signal-controlling genes, e.g., *p53* and the super-family of *Bcl-2*, many of which are localized in the nuclear, outer mitochondrial, and endoplasmic reticulum membranes thereby regulating the correct electrochemical homeostasis of the organelles. Cascade activation of caspases, which represent the actual death executioners, is responsible for much of the proteolysis occurring during apoptosis, whose targets are factors involved in the repair, fragmentation and duplication of DNA, in RNA splicing, in the maintenance of cytoskeletal integrity, and in cell division (Kidd, 1998; Wolf and Green, 1999; Hofmann, 1999, for reviews). Also stress response proteins, such as the molecular chaperones hsp60, hsc70, hsp70, and -90, have been recently proven to regulate key reactions of the apoptotic pathway, thus contributing in cell survival/death verdict (Samali and Orrenius, 1998; Garrido et al., 2001, for reviews). Apart from endogenous events, such as genomic DNA damage, a variety of extracellular ligands have been described to trigger apoptotic cell death (e.g., TNFα and Fas ligand), or even to exert a negative control (e.g., IGF-1 and GM-CSF), in some selected cytotypes (e.g., Nagata, 1997 for a review), and the number of them is destined to increase fastly due to the experimental efforts by laboratories worldwide.

It is generally acknowledged that the extracellular matrix, which provides a scaffold for connective tissue within organ architecture, contains a significant number of "informational" molecules whose interaction with scattered cells is of primary importance in the control of cellular proliferation, differentiation, and motility during histogenesis, for the maintenance of tissue homeostasis and in cancer development. One of the commonest features of most highly invasive tumors, such as the ductal infiltrating carcinoma (DIC) of the human breast, is the massive degradation of the basement membrane; on the other hand, remarkable qualitative and quantitative modifications of the interstitial collagen component have been described. Earlier data by Luparello et al. (1988) had shown that type V collagen, a "minor" component of normal breast stroma accounting for about 1% of total collagens, was over-deposited in the matrix of DIC, increasing up to 10% of extractable collagens. Subsequent "in vitro" experiments using the 8701-BC cell line, isolated from a biopsy fragment of primary DIC (Minafra et al., 1989), demonstrated that type V collagen was a poorly-adhesive substrate and that the subpopulation of 8701-BC cells able to attach onto it mainly utilized a non-integrin receptor recognized by an anti-67 kDa-elastin/laminin-binding protein antibody (Luparello et al., 1990, 1994; Minafra et al., 1992). On the other hand, adhesion of 8701-BC cells onto type V collagen resulted in a prominent slow-down of growth rate and inhibition of motility and invasion "in vitro" (Luparello et al., 1990, 1991; Pucci-Minafra et al., 1995); interestingly, more recent results (Pucci-Minafra et al., 2000) provided evidence that type V collagen was able to trigger oligonucleosomal DNA fragmentation, enhance the cellular uptake of ethidium bromide, a marker of loss of membrane integrity (Luparello et al., 2001), and up-regulate *m-calpain* whose product, a Ca^{++}-dependent neutral cysteine protease, has been found to be involved in cytoskeletal reorganization and apoptotic promotion in some model systems (Squier et al., 1994).

In light of these previous data suggesting that the growth-restraining activity shown by type V collagen on 8701-BC cells could be ascribed to an increase of cell death rate, we extended our investigation with the aim to (i) obtain further evidence by a specific assay that the death induced by type V collagen was of the apoptotic type, (ii) determine whether this collagen substrate could exert some effect on the expression level of selected genes coding for apoptosis regulators and for stress response proteins, and (iii) evaluate the extent of caspase activation in cells cultured on type V collagen.

MATERIALS AND METHODS
Cell cultures and substrates

8701-BC cell line, derived from DIC biopsy fragment (Minafra et al., 1989) was grown in 10% fetal calf serum (FCS)-containing RPMI 1640 medium (Gibco, Paisley/UK) plus antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells used were at early passages.

For the production of collagen substrates, type IV and V collagens, purchased from Sigma (St.Louis, MO), were dissolved in 0.5 M acetic acid, sterilized with chloroform, as already reported in Luparello et al. (1990), plated in tissue culture containers at the concentration of 10 µg/cm^2 for 48 h and exhaustively neutralized with PBS just before use. Type IV collagen was used as a control substrate, being a "natural" support for cells of epithelial origin.

Cell death detection system

For microscopic detection of apoptotic cells plated onto either type IV or type V collagen substrates, 8701-BC cells cultured for 48 h in collagen-coated 4-well Lab-Tek chamber slides (Nunc, Roskilde, Denmark) were assayed using the ApopTag Peroxidase In Situ Oligo Ligation kit (Serologicals Co., Norcross, GA). Briefly, fixed 8701-BC cells were submitted to "in situ" ligation reaction in the presence of either biotinylated oligo A, which contains a 3′-dA overhang, or biotinylated oligo B which is blunt-ended, the first oligo being more selective for the detection of "classical" apoptosis than other types of cell death. Labelled DNA was reacted with strepta-vidin-horseradish peroxidase and detected with diami-nobenzidine substrate provided within the kit. Nuclei were counterstained with methyl green. Cells incubated as above reported omitting ligation step served as negative controls.

Cell preparations were observed under an Olympus BX50 microscope equipped with a CoolSNAP-Pro Color digital camera (Media Cybernetics, Silver Springs, MD) and images recorded using Image Pro Plus software (Media Cybernetics). The percentage of ApopTag-positive 8701-BC cells was quantitated by counting the number of stained over total cells present in 10 random fields of the filter at a 200-fold magnification.

RNA extraction and reverse transcription

Isolation of total RNA from monolayers of 8701-BC cells grown in flasks for 48 h onto either type IV or V collagen substrate was carried out with TriPure reagent (Roche, Mannheim, Germany), according to manufacturer's instructions. Before the reverse transcription, 1–2 µg of total RNA were treated with RQ1 RNase-free DNase (Promega, Madison, WI). The cDNAs were synthesized in the presence of random 6-mer primers, using M-MLV RNase H⁻ point mutant reverse transcriptase (Promega).

Conventional and "semi-quantitative multiplex" (SM) PCR

PCR analysis was performed using 0.5 µM of appropriate sense and antisense primers (see Table 1) obtained from MWG Biotech AG, Ebersberg, Germany, 1 U RedTaq DNA polymerase (Sigma)/µl, 250 µM each of dNTPs, and 1 µl of the cDNA template obtained from total RNA. The thermal cycle used was a denaturation step of 94°C for 3 min, followed by 35−45 cycles of 94°C for 1 min, the appropriate annealing temperature for 1 min, and 72°C for 1 min. A final extension of the product was performed for 10 min at 72°C. PCR products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The primers for Bcl-xβ(Acc. nr. U72398) were chosen utilizing the "Primer Selection" software, available on-line at http://alces. med.umn.edu/rawprimer.html, and the identity of the amplification products was checked by sequencing.

For SM-PCR we followed a published protocol (Spencer and Christensen, 1999; Sirchia et al., 2003) in

TABLE 1. Sequence of primers used for PCR amplification

Transcript detected	Oligonucleotides (5′ → 3′)	Product size (bp)	Reference
Bcl-2	GCCTTTGTGGAACTGTACGGC GGCAGTAAATAGCTGATTCGACGTT	339	Luparello et al. (2003a)
Bcl-xLS	TTGGACAATGGACTGGTTGA GTAGAGTGGGATGGTCAGTG	780 (Bcl-xL) 591 (Bcl-xS)	Luparello et al. (2003a)
Bcl-xβ	CAGGTATTGGTGAGTCGGATCG GTCAGGTTTCCTCAACTATCAACGTC	217	
Bad	GTTCCAGATCCCAGAGTTTGAGC TTAAAGGAGTCCACAAACTCGTCACT	374	Luparello et al. (2003a)
Bax-α	ATGGACGGTCCGGGGAGCAGC CCCCAGTTGAAGTTGCCGTCAG	323	Luparello et al. (2003a)
Rip	TTTTGCACAGCAAAGACCTTACG AGCTGCTCAGAAGGTCGATCC	736	Luparello et al. (2003a)
Tiar	CCCCCTTTGGTAAAATATCGGAT GATTCCATGGTTGCCCGTATACT	629	Luparello et al. (2003a)
Cas	TGTTTTCATTGGTGGGCGTAA GCTGCAGTGAGCCATAATCGTACTA	373	Luparello et al. (2003a)
p53	ACAGCACATGACGGAGGTTGT CAGTGTGATGATGGTGAGGATGG	360	Luparello et al. (2003a)
Waf-1	AAGAACATGTGGACCTGTCA GGCTTCCTCTTGGAGAAGAT	170	Luparello et al. (2003a)
Dapk	GATGGCAACATGCCTATCGTG GATGAAGAGTCCTCGGTGCGTAT	266	Luparello et al. (2003a)
caspase-1	TTGATTGACTCCGTTATTC CTCTGCCGACTTTTGTTTC	285	Luparello et al. (2003a)
caspase-3	ATGGAGAACACTGAAAACTCA TTAGTGATAAAAATAGAGTTC	833	Soma et al. (1998)
caspase-4	GCTGTTTACAAGACCCACGTGG GTCGCTTCCATTTTATGAC	280	Soma et al. (1998)
caspase-5	CGGATCTGCTGCTTTATGAC AGGTTGCTCGTTCTATGGTG	423	Luparello et al. (2003a)
caspase-6	CGCAGATAGAGACAATCTTACCCG GACACACACAAAGCAATCGGC	147	Luparello et al. (2003a)
caspase-8	GCCTGCTGAAGATAATCAACGACTAT TTGATGATCAGACAGTATCCCCG	165	Luparello et al. (2003a)
caspase-9	TGGCTTCGTTTCTGCGAACTA GTTACTGCCAGGGGACTCGTC	679	Luparello et al. (2003a)
caspase-14	GATGCTCTGGAACACATGTTTCG CCCTTTGTTCTCCTCGACAGG	303	Luparello et al. (2003a)
hsp60	ATTCCAGCAATGACCATTGC GAGTTAGAACATGCCACCTC	306	Luparello et al. (2003b)
hsp70	TAACCCCATCATCAGCGGAC AACCGGAAAAAAAGCAAGTTCAGTA	260	Sconzo, personal communication
mthsp75	TGGCAGTTATGGAAGGTAAA AGCAATGACTTTGTCTTCTG	524	Luparello et al. (2003b)
hsp90α	AAAAGTTGAAAAGGTGGTTG TATCACAGCATCACTTAGTA	625	Luparello et al. (2003b)
hsp90β	AGAAGGTTGAGAAGGTGACAA AAGAGTAGAGAGGGAATGGG	641	Luparello et al. (2003b)
hsf-1	CCAGCAACAGAAAGTCGTCAA ATGTGCTGAGCCACTGTCGT	120	Luparello et al. (2003b)
hsf-2	ATGAAGCAGAGTTCGAACGTG GCTTTACAATTCCAGAGTCGATATG	246	Luparello et al. (2003b)
18 S	GGACCAGAGGCAAAGCATTTGCC TCAATCTCGGGTGGCTGAACGC	495	Spencer and Christensen (1999)

which the species of interest is co-amplified with 18S cDNA. The intensities of the band of interest, normalized for those of 18S, are plotted as a function of cycle number and exponential regression equations fitted to the curves are used to calculate the number of cycles necessary to reach a normalized intensity threshold value = 1 for each sample. The relative difference in abundance between two samples is taken as 2^n where n is the difference between the numbers of cycles required by the samples to reach the threshold. Two different RNA preparations from each experimental condition were pooled to make significative the differences between the expression levels, if any. PCR products were visualized by 2% agarose electrophoresis in the presence of ethidium bromide and, when required, band intensity evaluated with SigmaScan software (SPSS, Chicago, IL, USA).

Caspase activity detection system

Aliquots of 2×10^6 cells, mechanically scraped from collagen-coated flasks after 48 h of incubation, were lysed for 10 min in 50 μl of a chilled 50 mM Hepes buffer, pH 7.6, containing 150 mM NaCl, 5 mM EDTA, 10 mM PMSF, 10 mM 2-mercaptoethanol, 10% glycerol, 0.1% Triton X-100, and 10 μg/ml leupeptin, all purchased from Sigma. The lysate was centrifuged at 14,000 rpm for 1 min and the supernatant transferred to a new tube containing 50 μl of 2× reaction buffer (100 mM Hepes, pH 7.6, containing 50 mM EDTA, 0.005% Triton X-100, and 10 mM dithiotreitol) and 5 μl of either IETD-pNA,

WEHD-pNA, or LEHD-pNA chromogenic caspase substrates at 1 mM final concentration, all purchased from Alexis Biochemicals (San Diego, CA). Following 2 h of incubation at 37°C, the absorbance of the samples at $\lambda = 405$ nm was measured.

Statistics

Data are presented as mean $\pm$ s.e.m. of triplicate experiments performed three times; a software-assisted one-way ANOVA was performed (SigmaStat v.2.0, SPSS) and $P < 0.05$ was taken as the minimal level of statistical significance between samples.

RESULTS

In a first set of experiments 8701-BC cells, seeded and grown onto type IV and V collagen substrates as reported, were stained following the ApopTag protocol and examined under the microscope. At an overall observation, the morphological appearance of cells on the two different collagen substrates was markedly diversified, as already-described in previous publications (Luparello et al., 1990, 1991; Pucci-Minafra et al., 2000), with cells cultured onto type V collagen showing a less-spread cell morphology and a "packed" organization, and also a minor density of the culture, if compared with the counterpart grown as a flat monolayer onto type IV collagen substrate, despite a 2-fold amount of cells was initially seeded on the former substrate. In addition, as shown in Figure 1, more cells stained positively for apoptosis using the apoptosis-discriminating ApopTag oligo A; the percentage of Apoptag-positive cells was calculated, and the values for the cultures grown onto type IV or type V collagen substrates were $4.1 \pm 0.58\%$ and $60.4 \pm 4.2\%$ (when using oligo A), and $2.2 \pm 0.37\%$
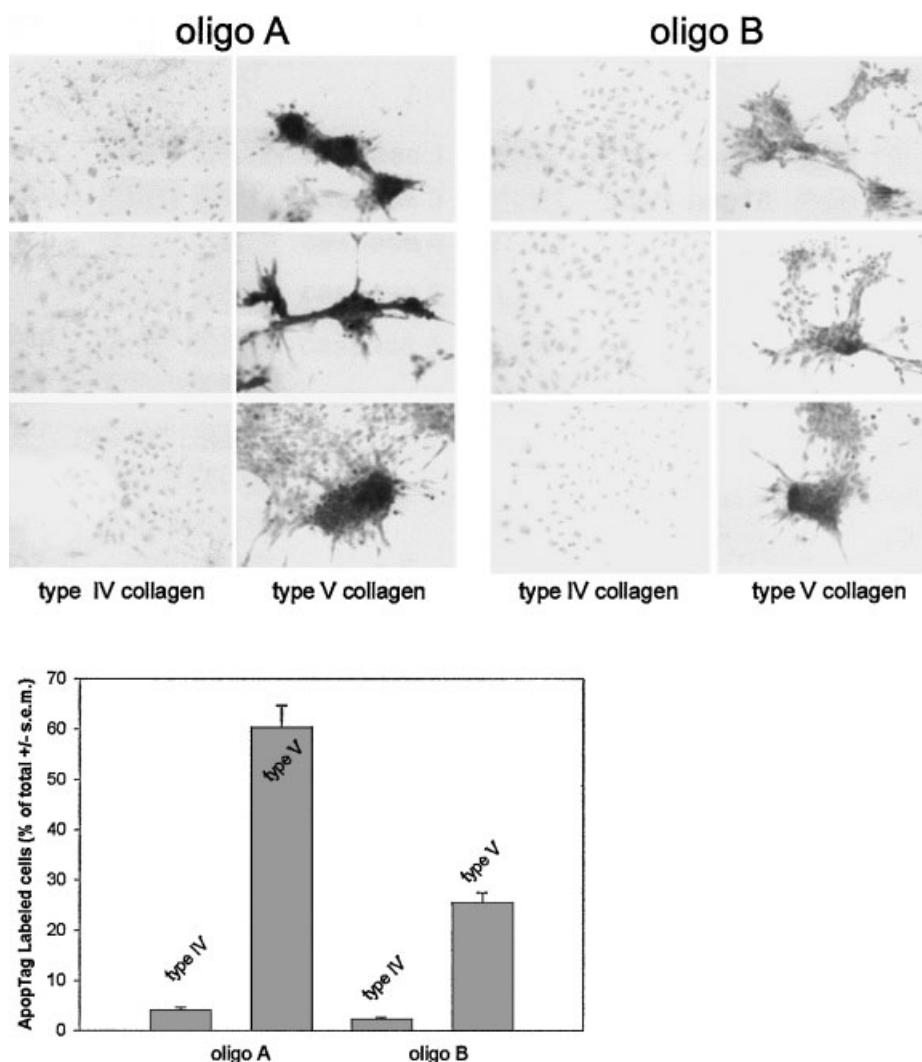

Fig. 1. Top: Part of micrographs of 8701-BC cells cultured onto either type IV or type V collagen substrates and submitted to Apoptag labeling using oligo A (one-base 3'-dT overhang-end detection) and oligo B (blunt-end detection). Microscopic magnification = 20×. Bottom: Histogram showing the percentage of Apoptag-positive cells after incubation of cell preparations with either oligo A or oligo B. The difference between the results obtained in the presence of either collagen substrate was significant ($P < 0.05$).

and $25.2 \pm 2\%$ (when using oligo B), respectively. In consideration of the already published data (Pucci-Minafra et al., 2000) reporting an effect of type V collagen on cellular uptake rate of ethidium bromide and on the onset of oligonucleosomal DNA fragmentation, taking previous and present data together, the concept that type V collagen, already known as a poor substrate for 8701-BC cell survival and growth, had apoptosis-promoting property was strongly supported.

Therefore, in a following set of experiments, we examined whether type V collagen was able to induce some changes in the pattern and level of gene expression of various apoptosis-agonist and -antagonist factors, stress response proteins, and caspases. On the basis of the observed low adhesion rate of 8701-BC cells onto type V collagen substrate (Luparello et al., 1990), all assays were performed on the sub-population of strongly-attached cells, i.e., detachable from the substrate only after trypsinization, after exhaustive washing of the culture to discard unattached or loosely-attached cells, in order to avoid the possible involvement of anoikis events (Frisch and Ruoslahti, 1997), due to loss of cell interaction with collagen support.

First, cDNA preparations from 8701-BC cells grown onto either collagen substrate were submitted to conventional PCR in the presence of primers specific for the selected genes; the part in Figure 2 shows that a positive signal was found for Bcl-2, Bcl-xL, Bcl-xS, Bcl-xβ, Bad, Tiar, p53, Dapk, and Waf-1 among the modulators, for all the genes for stress response factors tested, and for caspase-1, -5, -6, -8, -9, and -14; in particular, an amplification band for caspase-14 was evident only in the preparation from cells grown onto type V collagen. Conversely, no amplification band was observed for Rip, Cas, Bax-α, caspase-3 and -4, at least under the experimental conditions used.

Second, the cDNA preparations were submitted to SM-PCR to compare the expression levels of those genes coding for apoptosis controlling- and stress response factors, and caspases, selected on the basis of the previous results. From the results obtained, we found that type V collagen substrate was able to promote the up-regulation of Bcl-xS, Bad, and Dapk, by about 2.8-, 1.9-, and 2-folds, respectively, and the down-regulation of Bcl-2 and Bcl-xβ by about 2.6- and 14-folds (Fig. 3). Concerning the expression of stress response genes, hsf-1 and mthsp75 were up-regulated by about 2- and 3.7-folds in cells cultured onto type V collagen versus type IV collagen substrate, whilst hsp60 was down-regulated by about 7.9-folds in the same experimental condition (Fig. 4). Concerning the expression of caspases, adhesion of cells onto type V collagen induced the over-
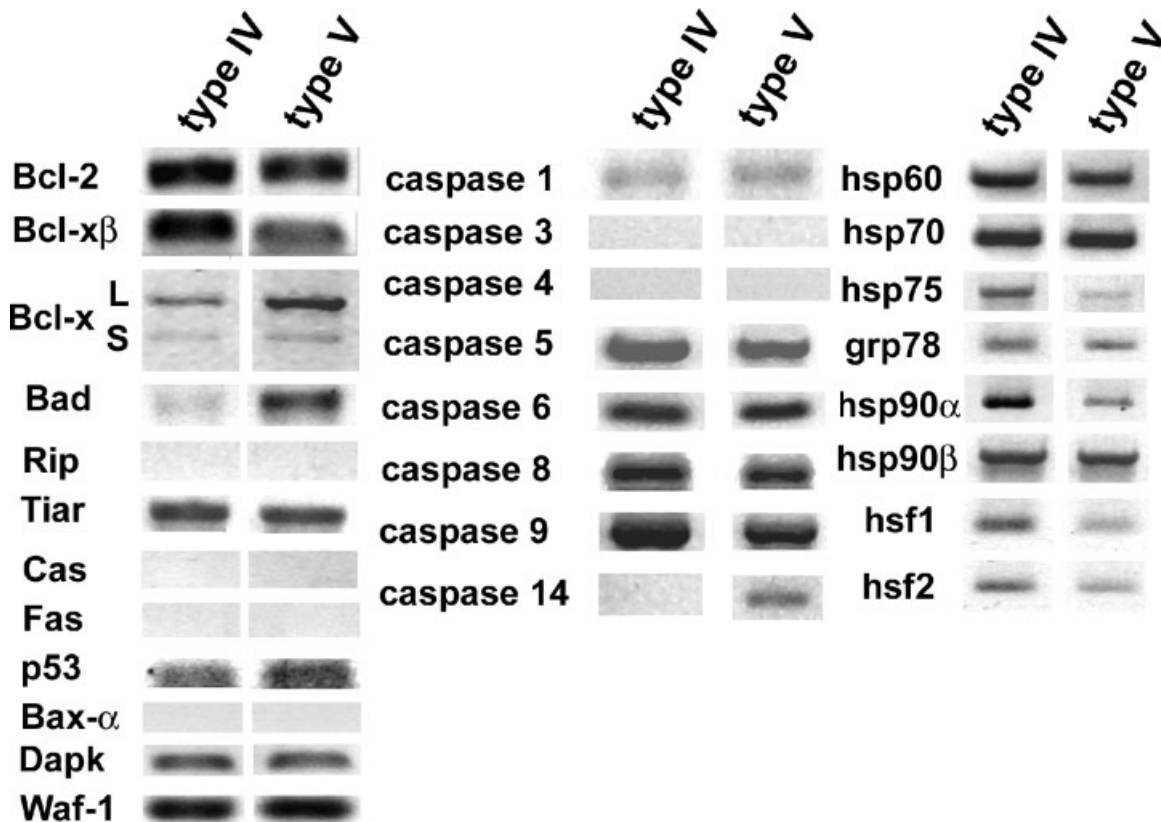

Fig. 2. Part of PCR analyses showing the presence of amplification products for Bcl-2, Bcl-xβ, Bcl-xL, Bcl-xS, Bad, Tiar, p53, Dapk, and Waf-1, for caspase-1, -5, -6, -8, -9 and for all the genes for stress response factors tested, in cDNA preparations from 8701-BC cells cultured onto both collagen substrates. Amplification product of caspase-14 was found only in preparations from cells plated onto type V collagen. No signal was found for Rip, Cas, Bax-α, caspase-3 and -4. Ethidium bromide stain.
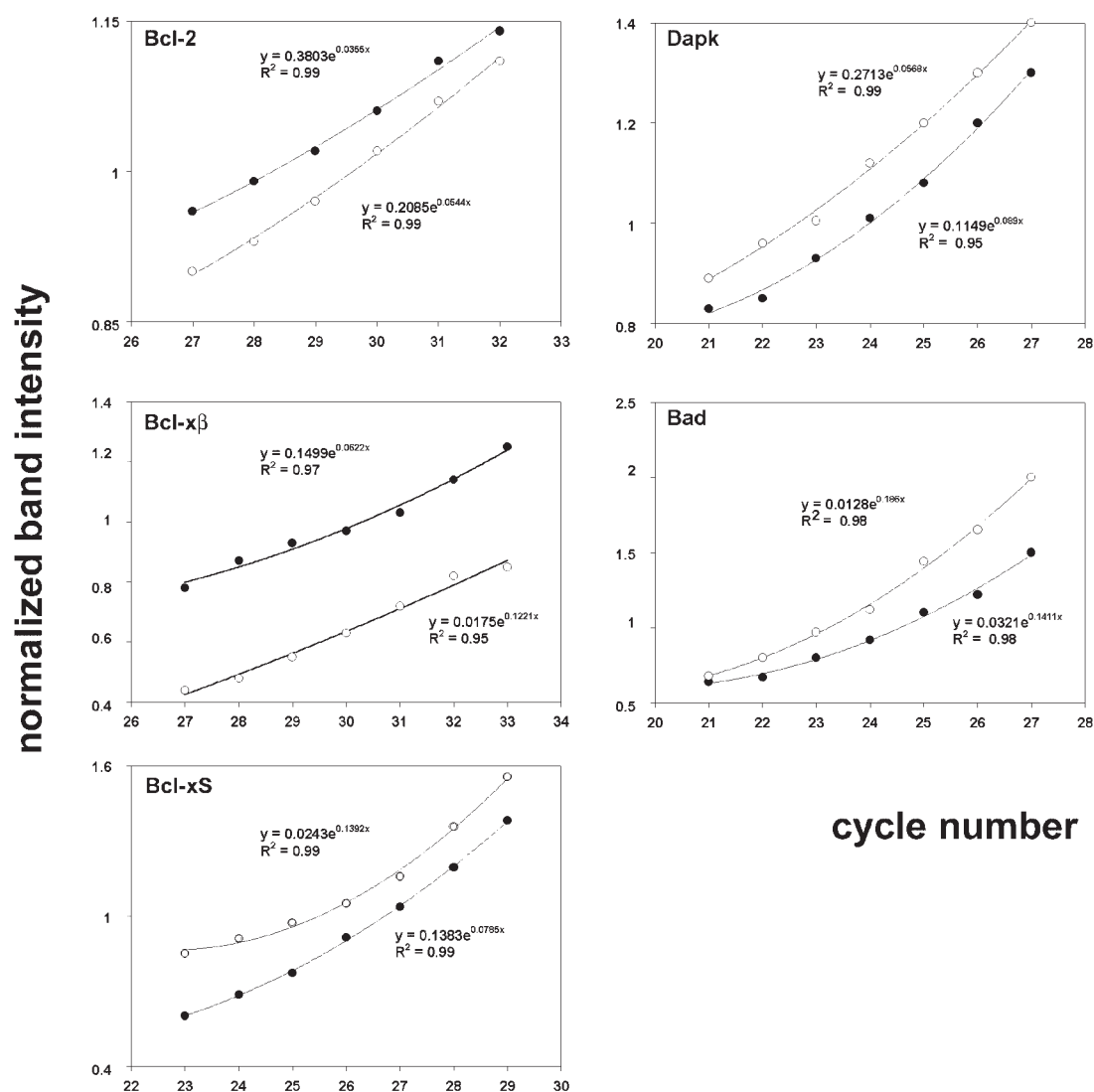
Fig. 3. SM-PCR for Bcl-2, Bcl-xβ, Bcl-xS, Dapk, and Bad. Representative plots of normalized data vs. cycle numbers fit with an exponential curve for 8701-BC cells cultured onto type IV (●) and type V collagen (○).

expression of caspase-1, -5, -8, and -9 by about 7.3-, 9.5-, 17-, and 28-folds, respectively (Fig. 5). No changes in the expression levels of *Bcl-xL*, *Tiar*, *Waf-1*, and *p53*, among the apoptosis-related genes; *hsf-2*, *hsp70*, *grp78*, *hsp90α*, and *-β*, among the stress-related genes; and *caspase-6*, among caspases, was found between 8701-BC cells grown onto type IV and type V collagen substrates (not shown). A synopsis of the data obtained and the functional properties of the products of the genes tested is shown in Table 2.

In light of the reported increase in the level of expression of some caspase genes, we wanted to check whether this event was related to the augmented activity of the same proteases. To this purpose, lysates of cells grown onto either collagen substrates were prepared and assayed for their ability to cleave chromogenic substrates containing tetrapeptide sequences mainly recognized by caspase-1 and -5 (WEHD-pNA),

caspase-8 (IETD-pNA), and caspase-9 (LEHD-pNA) (Thornberry et al., 1997). As shown in Figure 6, the presence of type V as culture substrate for 8701-BC cells determined an increase of enzymatic activity of about 18-, 13-, and 14-folds versus cells grown onto type IV collagen, thus indicating that the former collagen species was able to trigger a transcriptional up-regulation as well as a strong activation of caspase proteases.

DISCUSSION

Although the structural and molecular characteristics of type V collagen have been described extensively since more than a decade (Bateman et al., 1996 for a review), the biological role of this "minor" component of the extracellular matrix and the effect of its interactions with different cytotypes are still poorly understood. From literature data, it appears unlikely to define a generic and unique role played by type V collagen since
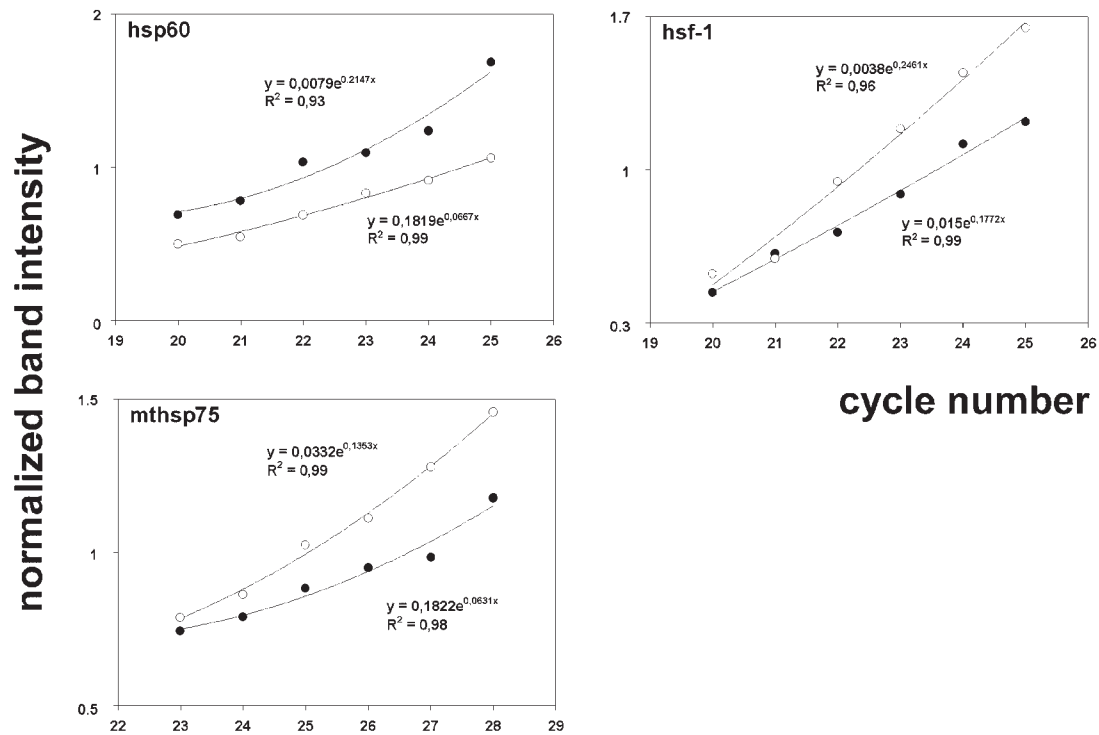
Fig. 4. SM-PCR for hsp60, mthsp75, and hsf-1. Representative plots of normalized data vs. cycle numbers fit with an exponential curve for 8701-BC cells cultured onto type IV (●) and type V collagen (○).
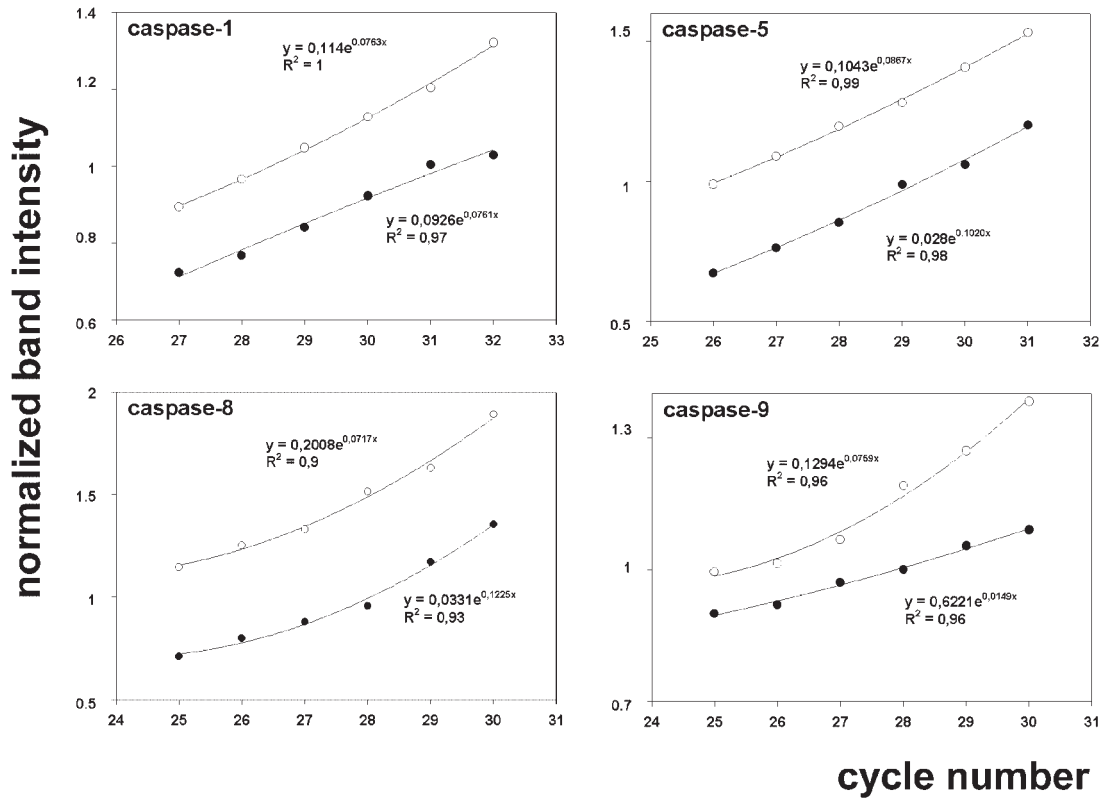

Fig. 5. SM-PCR for caspase-1, -5, -8, and -9. Representative plots of normalized data vs. cycle numbers fit with an exponential curve for 8701-BC cells cultured onto type IV (●) and type V collagen (○).

TABLE 2. Apoptosis-related and stress protein genes studied in the present study

Gene product	Function	Expression level ratio (type V coll.: type IV coll.)
Bcl-2	Anti-apoptotic 26/30 kDa-component of nuclear, mitochondrial and endoplasmic reticulum membrane; founding member of Bcl-2 super-family, binds Bax and Bak (Newton and Strasser, 1998).	1:2.6
Bcl-x	Member of Bcl-2 super-family; L form of 29-kDa inhibits apoptosis, S form of 21 kDa promotes apoptosis, β form is homologue to the murine counterpart which prevents cell death; binds Bax and Bak (Gonzalez-Garcia et al., 1995; White, 1996; Ban et al., 1998).	No change (L form), 2.8:1 (S form), 1:14 (β form)
Bad	25 kDa-pro-apoptotic member of Bcl-2 super-family; binds Bcl-2 and Bcl-xL (White, 1996).	1.9:1
Bax-α	21 kDa-pro-apoptotic member of Bcl-2 super-family; binds Bcl-2 and Bcl-xL (White, 1996).	Undetected
Rip	(Receptor-interacting protein) pro-apoptotic 74 kDa-protein which interacts weakly with the p55 tumor necrosis factor receptor intracellular domain (Grimm et al., 1996).	Undetected
Tiar	(TIA-1-related protein) pro-apoptotic 42 kDa-RNA-binding factor (Kawakami et al., 1992).	1:1
Cas	(Cellular apoptosis-susceptibility protein) pro-apoptotic 100 kDa-human homologue of yeast chromosome-segregation protein (CSE-1) (Brinkmann, 1998).	Undetected
Waf-1	Pro-apoptotic 21 kDa-protein which mediates the tumor-suppressor properties of p53 (Özçelik et al., 1995).	1:1
p53	Pro-apoptotic 53 kDa-chromatin- and nuclear matrix-associated factor (Cotter and McCarthy, 1994).	1:1
Dapk	(Death-associated protein kinase) pro-apoptotic 160 kDa-Ca^{++}-calmodulin-regulated serine/threonine kinase (Levy-Strumpf and Kimchi, 1998).	2:1
hsp60	(60 kDa-heat shock protein) anti-apoptotic mitochondrial chaperonin that binds Bax and Bak (Kirchhoff et al., 2002).	1:7.9
hsp70	(70 kDa-heat shock protein) cytosolic/nuclear chaperonin whose overexpression protects cells from stress-induced apoptosis (Garrido et al., 2001).	1:1
mthsp75	(mitochondrial 75 kDa-heat shock protein) chaperonin which is up-regulated during anticancer drug-induced apoptosis (Kim et al., 1999).	3.7:1
grp78	(78 kDa glucose-regulated stress protein) endoplasmic reticulum- associated chaperonin whose induced expression is correlated to resistance to apoptotic death (Fernandez et al., 2000).	1:1
hsp90 α/β	(90 kDa-heat shock protein) isoforms of a cytosolic chaperonin which shows predominant, but not exclusive, anti-apoptotic effects (Garrido et al., 2001).	1:1
hsf-1	(heat shock factor-1) transcription factor for heat shock genes whose DNA-binding activity is increased during anticancer drug-induced apoptosis (Kim et al., 1999).	2:1
hsf-2	(heat shock factor-2) transcription factor for heat shock genes presumably endowed with anti-apoptotic activity, at least on selected mouse cytotypes (Kallio et al., 2002).	1.1
caspase-1	Protease involved in both cytokine-processing and apoptosis (e.g., Kuida et al., 1995); prototypic substrates are PARP, pro- caspase-1, -2, -3 and actin (Kidd, 1998).	7.3:1
caspase-3	Small prodomain-apoptotic executioner, active on PARP, gelsolin and actin (Kothakota et al., 1997; Stennicke and Salvesen, 1997).	Undetected
caspase-4	caspase-1-related enzyme involved in the promotion of fibroblast apoptosis (Munday et al., 1995).	Undetected
caspase-5	caspase-1-related enzyme involved in Fas-mediated apoptosis (Krippner-Heidenreich et al., 2001).	9.5:1
caspase-6	Small prodomain-apoptotic executioner; active on PARP and nuclear lamins (Stennicke and Salvesen, 1997; Wolf and Green, 1999).	1:1
caspase-8	Apoptotic initiator which is able to proteolytically activate other downstream caspases (Stennicke and Salvesen, 1997).	17:1
caspase-9	Apoptotic initiator which is able to proteolytically activate other downstream caspases and cleave PARP (Wolf and Green, 1999).	28:1
caspase-14	Small prodomain-enzyme expressed in embryonic tissue and involved in terminal differentiation of keratinocytes and apoptosis of breast cancer cells (Hu et al., 1998; Pistritto et al., 2002).	Type V collagen only

the cellular responses evoked by this collagen substrate are multiple, and sometimes opposite, according to the cell type examined. In fact, collagen type V has been proven to represent an optimal substrate for the attachment and growth of hepatocytes (Takai et al., 2001), Schwann cells (Chernousov et al., 2001), normal smooth muscle cells (Grotendorst et al., 1981; Leushner and Haust, 1985), and of some tumor cell types (Ruggiero et al., 1994); conversely, an anti-adhesive and anti-proliferative role played by this collagen species on neurons (Chernousov et al., 2001), various epithelial and endothelial cell lines (Parekh et al., 1998; Underwood et al., 1998), and breast cancer cells (Luparello et al., 1990, 1991, 2003a; Pucci-Minafra et al., 2000) has been described.

In the present study we extend previous analyses reported by Pucci-Minafra et al. (2000) and demonstrate that type V collagen is able to impair survival of 8701-BC breast cancer cells by promoting a caspase-dependent apoptotic type of death, providing also some information at gene expression level. In fact, our results report the up-regulation of apoptotic initiators caspase-8 and -9, and of the apotosis-involved caspase-1 and the related caspase-5 and -14, as well as a similar increase of the activity of some of the enzyme products. Further studies will be required to determine the precise intracellular pathway through which this collagen species accomplishes its lethal effects; however, the data here presented allow to make the following principal comments.

First, the analysis of the expression of the genes for apoptotic modulators indicate a down-regulation of two anti-apoptotic genes, *Bcl-2* and of the β form of *Bcl-x*, and the up-regulation of the pro-apoptotic *Bcl-xS* and *Bad*,
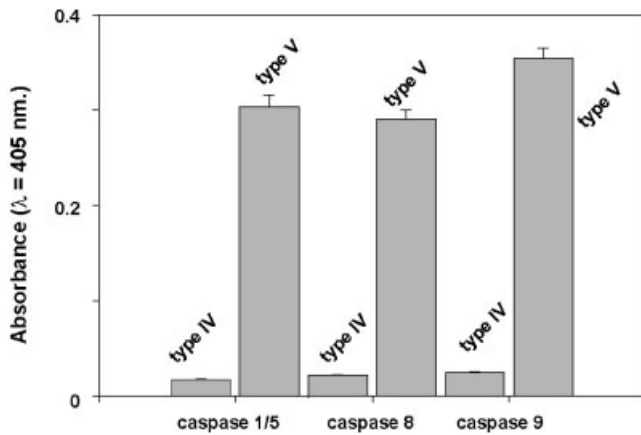
Fig. 6. Histogram showing the increase of the enzymatic activity of caspase-1/-5, -8, and -9 in lysates of 8701-BC cells grown onto type V vs. type IV collagen substrate by chromogenic assay. The difference between the results obtained in the presence of either collagen substrate was significant ($P < 0.05$).

which per se could be a key factor for the onset of programmed cell death (Newton and Strasser, 1998). Moreover, the up-regulation of Dapk appears particularly interesting: its gene product is a Ca^{++}/calmodulin-dependent serine/threonine kinase, acting on several factors involved in apoptosis, such as DAP-1, a proline-rich cytoplasmatic protein; DAP-3, a nucleotide-binding protein; and DAP-5, homologous to the translation initiation factor eIF4G, in response to Fas, TNFα, γ-interferon and also poorly-adhesive extracellular matrix substrates (Levy-Strumpf and Kimchi, 1998; Cohen and Kimchi, 2001). Dapk concentrates on actin microfilaments (Inbal et al., 1997), and was proven to induce the disassembly of stress fibres, as well as the suppression of integrin functions and integrin-mediated survival signals (Wang et al., 2002); thus, it is conceivable that type V collagen-dependent up-regulation of Dapk may contribute to the modification of morphological appearance and to the prominent decrease of cell spreading observable when 8701-BC cells are seeded onto this collagen substrate. Validation of this hypothesis awaits further studies.

Second, literature reports demonstrate that the increase of hsf-1 DNA-binding activity with consequent and selective up-regulation of mitochondrial hsp75 is responsible of apoptotic death of tumor cells treated with anticancer drugs and that, conversely, hsp60 is able to play a prominent anti-apoptotic role, by binding and inhibiting apoptosis-promoting cytosolic factors (Kim et al., 1999; Kirchhoff et al., 2002). Although determination of the actual activation state of hsf-1 following its over-expression will require further analysis, and the possible concomitant intervention of other stress response factors (i.e., hsp90 and heat shock factor-binding protein-1) in the control of hsf-1 function (Luparello et al., 2003b) remains to be determined, the results obtained on stress protein gene expression indicating the up-regulation of hsf-1 and mthsp75 and a marked down-regulation of hsp60 are a further support to the hypothesis that type V collagen acts as an apoptotic inducer for 8701-BC cells.

Third, our work examines the array of transcripts and the enzymatic activity displayed by cells after 48 h from seeding onto type V collagen substrate, that is the minimum time required to establish a stable adhesion by 8701-BC cell subpopulations able to attach onto it (Luparello et al., 1990). It is known that the expression of caspases and apoptosis-linked factors is subjected to stringent temporal controls at transcriptional levels, and that also protease activity undergoes temporal induction (e.g., Benjamin et al., 1998; Harrison et al., 2001). Therefore, present data may conceivably represent a "photograph" of one stage of a highly complex and dynamic process, which will necessarily need additional analyses at anterior or posterior times, in which we expect to observe distinctive patterns of gene expression and caspase activation. Noteworthy, in our model system type V collagen appears to switch-on the expression of *caspase-14*, whose message cannot be found in cDNA preparations from cells cultured onto type IV collagen. This result is consistent with findings from Pistritto et al. (2002) indicating that epithelial cells, including those from breast, up-regulate caspase-14 in conditions of lack of correct matrix interactions, and provide an experimental model suitable for the study of molecular events regulating *caspase-14* gene expression.

Interestingly, in a previous study we have also found that another breast cancer cell line, i.e., T47-D, undergoes apoptosis in response to the presence of type V collagen (Luparello et al., 2003a) but data comparison reveals a pattern of up- and down-regulation of apoptosis-linked genes somewhat different from that of 8701-BC cells (see Table 3 for a synopsis), suggesting the existence of a certain degree of heterogeneity in the intracellular transduction of type V collagen-driven death signal among cell lines of similar origin (i.e., derived from DIC). Nonetheless, it is worth mentioning that the tissue source of the two cell lines was diverse (i.e., primary site for 8701-BC cells, pleural effusion for T47-D cells), and, therefore, the model system 8701-BC

TABLE 3. Expression level ratios of apoptosis-related genes in 8701-BC and T47-D breast cancer cells cultured onto type V collagen vs. type IV collagen substrates*

Gene	Cell line	
	8701-BC	T47-D[a]
Bcl-2	1:2.6	1:3
Bcl-xL	1:1	1:1
Bcl-xS	**2.8:1**	**1:1**
Bad	**1.9:1**	**Undetected**
Tiar	1:1	1:1
Cas	**Undetected**	**5.3:1**
p53	**1:1**	**2.8:1**
Dapk	2:1	2.8:1
Waf-1	**1:1**	**8.6:1**
caspase-1	**7.3:1**	**2:1**
caspase-5	**9.5:1**	**5.1:1**
caspase-6	1:1	1:1
caspase-8	**17:1**	**1:1**
caspase-9	**28:1**	**1:1**
caspase-14	**only on type V collagen**	**2.4:1**

*The more representative differences in the modulation of gene expression between the two model systems are marked in bold.
[a]Luparello et al. (2003a).

line-type V collagen is the only available mimicking the interactions between an original tumor cell population, exempt of metastatic selection, and this collagen species whose over-deposition was found in primary specimens (Luparello et al., 1988). In addition, previous results also showed that Hs578T and MDA-MB231 breast cancer cell lines which come from specimens of carcinosarcoma and non-ductal adenocarcinoma of the breast, respectively, were insensitive to the inhibitory effect of type V collagen (Luparello et al., 2003a), thus suggesting that such effect could be specifically selective for the ductal histotypic variant of mammary tumor, conceivably due to particular arrays of surface receptors exposed and/or intracellular signalling present. Further studies will be required to confirm this hypothesis.

In conclusion, present data (i) show that type V collagen is an efficient viability-inhibitor and apoptotic-promoter for 8701-BC cells, and (ii) validate this cell line as a suitable "in vitro" model for further and more detailed studies on the molecular mechanisms of the death response induced by this collagen species on primary DIC cells.

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