Type V Collagen and Protein Kinase C η Down-Regulation in 8701-BC Breast Cancer Cells

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We previously reported that ductal infiltrating carcinomas (d.i.c.) of the human breast display profound modifications of the stromal architecture, associated with anomalous collagen composition. Among the major alterations observed in the interstitial collagen, the relative increase of type V collagen content was detected. When type V collagen was used as an ''in vitro'' substrate for 8701-BC d.i.c. cells, it appeared able to restrain cell growth, inhibit cell motility and invasion ''in vitro'', and modify the expression levels of genes coding for apoptosis factors, caspases and stress response proteins. In the present paper we demonstrate that type V collagen induces the down-regulation of *protein kinase C η*, an event that may be, at least in part, responsible of the previously-reported modifications of cell morphology and growth rate, and that appears to be involved in the already-observed changes of expression levels of genes encoding for anti- (*Bcl-2)* and pro-apoptotic factors (*Bad*, *Dapk*, *Bcl-Xs*) and enzymes (*caspase 5* and *8*).

Key words: differential display PCR; gene expression; apoptosis

INTRODUCTION

It is generally acknowledged that the extracellular matrix (ECM) is not to be regarded as a mere passive scaffold for connective tissues within organ architecture, but that cell-ECM interactions may influence a number of biological activities. The dynamic space-temporal interactions of cells with their ECM are indeed known to drive embryonic morphogenesis and to maintain tissue homeostasis in adult organs. Cell behaviors that are modulated by (or associated to) cell-matrix interactions include growth, differentiation, apoptosis, motility, signal transduction and gene expression (e.g., [1,2] for reviews). Local perturbation of ECM composition and architecture following pathologic events, may result in a selectively-reprogrammed cell behavior, through a cascade of signals difficult to predict a priori.

The ductal infiltrating carcinoma (d.i.c.) is one of the most common invasive histotypes of breast cancer, as well as that endowed with the highest incidence and lethality. It is known that the ECM of d.i.c.-affected breast undergoes to desmoplastic reaction commonly characterized by the over-deposition of a dense fibrous tissue containing mainly collagen among the newly-synthesized ECM components [3]. The major aspects of collagen changes recorded in d.i.c. are the over-deposition of α1(I)-like chains, (mainly assembled as an homotrimeric $[\alpha1(I)]_3$ variant named onco-foetal/laminin binding, i.e., OF/LB) and of type V collagen which is normally expressed at very low levels in adult tissues [4]. Type V collagen, which belongs to the category of the fibril-forming collagens, can be deposited as different isoforms with distinct chain composition, such as $\alpha1(V)_2$ α2(V), which is the most common, $\alpha1(V)_3$ and α1(V)α2(V)α3(V) and can be predominantly found in pericellular areas ([5] for review). The cellular responses elicited by this collagen appear heterogeneous, suggesting differences among the model systems examined (e.g., tissue and cell types). For example, adhesion- and motility-promoting activity has been observed for glomerular endothelial cells, Schwann cells, and osteosarcoma cells [6–8]; on the other hand, anti-adhesive, anti-proliferative and/or anti-locomotory effects have been observed on cytotypes such as fibroblasts, colorectal adenocarcinoma cells, and lung epithelial cells [9–11].

Biological assays performed with the neoplastic cell line 8701-BC, isolated from a primary d.i.c., demonstrated that type V collagen is a poorly-adhesive substrate also for these cells, which utilize mainly the 67 kDa-laminin/elastin receptor for its recognition [12]. In addition, the 8701-BC subpopulation able to attach onto type V collagen substrate exhibits a number of programmed death-

Abbreviation: ECM, extracellular matrix; d.i.c., ductal infiltrating carcinoma; DD-PCR, differential display-polymerase-chain-reaction; PKCη, protein kinase C η; *Dapk*, death-associated protein kinase; asODN, antisense oligonucleotide; SM-PCR, semi-quantitative ''multiplex'' PCR; TBS-T, Tris-buffered saline/0.05% Tween-20; ERK, extracellular signal-regulated kinase.

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related features, such as slow-down of growth rate, inhibition of motility and invasion ''in vitro,'' loss of membrane integrity, oligonucleosomal DNA fragmentation, changes in the expression levels of genes coding for apoptosis-linked factors, calpain, caspases and stress response proteins, and enhancement of caspase enzymatic activity [13–17]. These data collectively indicate that 8701-BC cell line may represent a suitable ''in vitro'' model for further and more detailed studies on the molecular mechanism of the death response induced by the ''natural'' component type V collagen on d.i.c. cells. Interestingly, a more elevated deposition of type V collagen in the ECM of lung cancer was also proven to be significantly associated with patient survival time [18].

In consideration of the potentially-relevant implications of restraining micro-environmental influences on tumor progression, we set out experiments to shed more light on the effects at a molecular level of 8701-BC cells/type V collagen interplays. In particular, we wanted to expand the knowledge on type V collagen-dependent genes expressed by 8701-BC cells [16,19], using the ''differential display'' (DD)-polymerase chain reaction (PCR) described by Sokolov and Prockop [20] which allows the identification of cDNAs from differentially-expressed genes by analysis of their internal sequences after reverse transcription with random hexamers and amplification in the presence of arbitrary primers. As a control substrate we utilized type IV collagen, a basement membrane component and therefore a ''physiologic'' support for cells of epithelial origin, whose promoting effect on survival and proliferation of breast cancer cells, including 8701-BC, has been already recognized [16,21].

Here we demonstrate that type V collagen induces the down-regulation of *protein kinase C η* (*PKCη*), an event that may be, at least in part, responsible of the phenotypic modifications reported by Luparello and Sirchia [16], seemingly acting upstream to the already-observed changes of expression levels of genes encoding for anti- (*Bcl-2*) and pro-apoptotic factors (*Bad*, *Dapk, Bcl-Xs*) and enzymes (*caspase 5* and *8*).

MATERIALS AND METHODS

Collagens

Type IV and V collagens were purchased from Sigma (St.Louis, MO). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of Type V collagen preparation, performed as reported by Luparello et al. [13], revealed that it consisted almost exclusively of the $\alpha1(V)_2\alpha2(V)$ isoform (data not shown). Collagens were dissolved in 0.5 M acetic acid, sterilized with chloroform, plated in 25 cm^2 flasks at the concentration of 10 $\mu g/cm^2$ for 48 h and exhaustively neutralized with phosphate-buffered saline (PBS) just before cell culturing.

Cell Cultures and Transfection Experiments

8701-BC cells, derived from a primary d.i.c. [22] were routinely cultured in RPMI 1640 medium, supplemented with 10% foetal calf serum (Invitrogen, Paisley, UK) and antibiotics/antimycotics, in a 5% CO_2 atmosphere. Trypsinized 8701-BC cells, resuspended in serum-free medium, were plated in substrate-coated flasks at the concentration of either $6 \times 10^5/cm^2$ (on type IV collagen) or $1.2 \times 10^6/cm^2$ (on type V collagen), because of the different adhesion rate onto the two substrata [13], and allowed to grow for 48 h. A directed antisense strategy was applied to down-regulate *PKCη* expression in 8701-BC cells grown onto type IV collagen substrate, as described by Sonnemann et al. [23]. The sequence of PKCη antisense oligonucleotide (asODN) was 5′-AGGCCCGTACAGCATTTCCT-3′, whereas that of the ''scrambled'' control asODN was 5′-TAACCACTCAGCCTAGCGTC-3′. Either asODN was delivered to cells as a complex with Lipofectin (Invitrogen), according to manufacturer's instructions, in serum- and antibiotic/antimycotic-free medium. Before undergoing to RNA extraction, cells were observed under phase-contrast microscope and photographed.

Cell Number Evaluation

Equal amounts of control and transfected 8701-BC cells were grown on collagen-coated 6-well dishes (Nunc, Roskilde, DK) for 48 h. Cell numbers were measured by counting in a Bürker chamber after trypsinization. Data are presented as mean $\pm$ SEM of quadruplicate experiments. A Student's *t*-test was used and $P < 0.05$ was taken as the minimal level of statistical significance of the differences between treated and control samples.

ApopTag Staining

Control and transfected 8701-BC cells were cultured in 4-well chamber slides (Nunc) and assayed using the ApopTag Peroxidase ISOL Apoptosis Detection Kit (Millipore, Billerica, MA) as reported in Ref. [16]. Essentially, ''in situ'' ligation reactions were performed with biotinylated oligo A, which contains a 3′-dA overhang selective for the detection of apoptotic cells. Labeled DNA was reacted with streptavidin-horseradish peroxidise and detected with diaminobenzidine substrate provided in the kit. Nuclei were counterstained with methyl green. Negative controls omitting ligation step were incubated in parallel. 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).

RNA Extraction and Reverse Transcription

Isolation of total RNA from monolayers of 8701-BC cells was carried out with TRIzol reagent (Invitrogen), according to manufacturer's instructions. Before the reverse transcription, the total RNA were treated with RQ1 RNase-free DNase (Promega, Madison,WI) and its quality and integrity checked through agarose gel electrophoresis in denaturing conditions. The cDNAs were synthesized from 1–2 μg of DNase-treated RNA in the presence of random 6-mer primers, using SuperScript II reverse transcriptase (Invitrogen), and their quality was checked by amplification of ''housekeeping'' 18S cDNA.

DD-PCR

For differential expression analysis, DD-PCR experiments were performed as already reported (e.g., [19,24]) using the arbitrary 10-mer primers designed by Sokolov and Prockop [20], in combinations of two. The PCR amplification was carried out using 25 pmol of each of two primers, 1–2 μl of the cDNA template and 3.6 U of AmpliTaq DNA Polymerase, Stoffel fragment (Applied Biosystems/ Life Technologies, Carlsbad CA), in 50 μl of the appropriate reaction mixture. The thermal cycle used was a denaturation step of 94.5°C for 3 min, followed by 45 cycles of 94.5°C for 1 min, 34°C for 1 min, 72°C for 1 min, and a final extension of the product for 10 min at 72°C.

After PCR amplification, 8 μl of the amplification products were electrophoresized in a non-denaturing 6% polyacrylamide gel in a sequencing apparatus at constant 55 W and the band pattern visualized via silver staining. Evaluation of band size was performed with SigmaGel software (SPSS, Blairgowrie, UK). For re-amplification of differentially-displayed bands, the silver-stained gel was exhaustively washed with double-distilled water and the band of interest carefully scratched from the gel with a sterile syringe needle and used as template for PCR amplifications performed as described in the following section. Several cycles of amplification and electrophoresis were repeated until a single pure band was visualized in the gel and eluted using Ultrafree DA filter columns (Millipore, Bedford, MA).

The purified PCR product was subsequently cloned using the pGEM-T Easy vector system (Promega) and JM109 competent cells, high efficiency (Promega); the sequence of the inserts contained in the recombinant plasmid DNA, isolated with High Pure Plasmid Isolation kit (Roche, Basel, Switzerland), was determined by MWG Biotech sequencing service. DNA sequence similarity was searched with the BLAST algorithm [25] available on-line.

Conventional Polymerase Chain Reaction (PCR)

PCR analysis was performed using 2.5 μM of appropriate sense and antisense primers, 1 U RedTaq DNA polymerase (Sigma)/μl, 200 μM each of deoxyribonucleotides diphosphates, and 1 μl of the cDNA template obtained from total RNA. The latter one was omitted in negative controls. All the primers used were from Luparello and Sirchia [16] except for those for PKCη designed with the Primer3 software available on-line, and specific for a 260 bp sequence from bases 511–771 of the PKCη coding sequence (Acc. nr. M55284). The thermal cycle used was a denaturation step of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, the appropriate annealing temperature for 30 s, and 72°C for 30 s. A final extension of the product was performed for 5 min at 72°C. PCR products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The identity of the PKCη PCR product was confirmed by sequencing.

Semi-Quantitative ''Multiplex'' (SM)-PCR

SM-PCR was carried out as previously reported (e.g., [16,21,24]). Essentially, the cDNA species of interest was co-amplified with cDNA from 18S rRNA over a range of cycles, followed by 2% agarose electrophoresis and ethidium bromide stain. Cycle profile was a denaturation step of 94.5°C for 3 min, followed by cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final extension of the product for 5 min at 72°C. Cycles were limited to the minimum necessary for detection. The intensities of the bands of interest, evaluated with SigmaScan software, were normalized for those of 18S, and plotted as a function of cycle number. Exponential regression equations fitted to the curves were 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 was 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 more significant the differences between the expression levels, if any.

Protein Extraction and Western Blot

Control and treated 8701-BC cells were scraped from the substrate and lysed with 100 μl of prewarmed lysis buffer (40 mM Tris–HCl, pH 6.8, containing 1% sodium dodecyl sulphate, 1% glycerol, 1% β-mercaptoethanol, and 0.001% bromophenol blue). Aliquots of the extracts, normalized for protein content, were subjected to SDS-PAGE (7.5% acrylamide) and blotted onto Immobilon P filters (Millipore) in a Bio-Rad transfer apparatus. After

blocking for 1 h with Tris-buffered saline/0.05% Tween-20 (TBS-T) containing 5% nonfat dry milk at room temperature, filters were incubated for 1 h with either anti-PKCη (Novus Biologicals, Littleton, CO; final dilution 1:1,000), or anti-β-actin (A2066 from Sigma; final dilution 1:100) in TBS-T containing 1% nonfat dry milk. After being washed six times for 5 min each with TBS-T, the filters were incubated for 1 h with the peroxidase-conjugated secondary antibody dissolved in TBS-T containing 1% nonfat dry milk and washed six times for 5 min each with TBS-T, prior to detection by the SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL), following manufacturer's recommendations.

RESULTS

Type V Collagen Down-Regulates PKCη in 8701-BC Cells

In order to search for putative molecular markers linked to the lethal phenotype induced by type V collagen, in a first set of assays we submitted cDNA preparations, obtained from 8701-BC cells grown onto either type IV or type V collagen substrata, to DD-PCR as described. Among the several differentially-displayed bands present in the gel, we focused our attention on an amplification band of about 160 bp, obtained utilizing the BS57/BS58 combination of arbitrary primers, which was present only in the electrophoretic lane corresponding to the cDNA preparation from cells grown onto type IV collagen substrate (Figure 1A). The differentially-displayed cDNA fragment was purified by excision from the gel and several cycles of PCR and electrophoresis, cloned and submitted to sequencing. Using BLASTN software, homology (score = 207 bits, expect = 6e−51) was found in the non-redundant nucleic acid sequence database (nr–nt) between the sequence obtained and that of region 324–474 of the coding sequence for Homo sapiens *PKCη* (Acc. nr. M55284), as deposited by Bacher et al. [26].

We then checked the differential expression of *PKCη* by PCR amplification in the presence of specific primers, designed using the Primer3 software available on-line. As shown in Figure 1B, after the PCR amplification we found a product of the expected size (180 bp) in both experimental

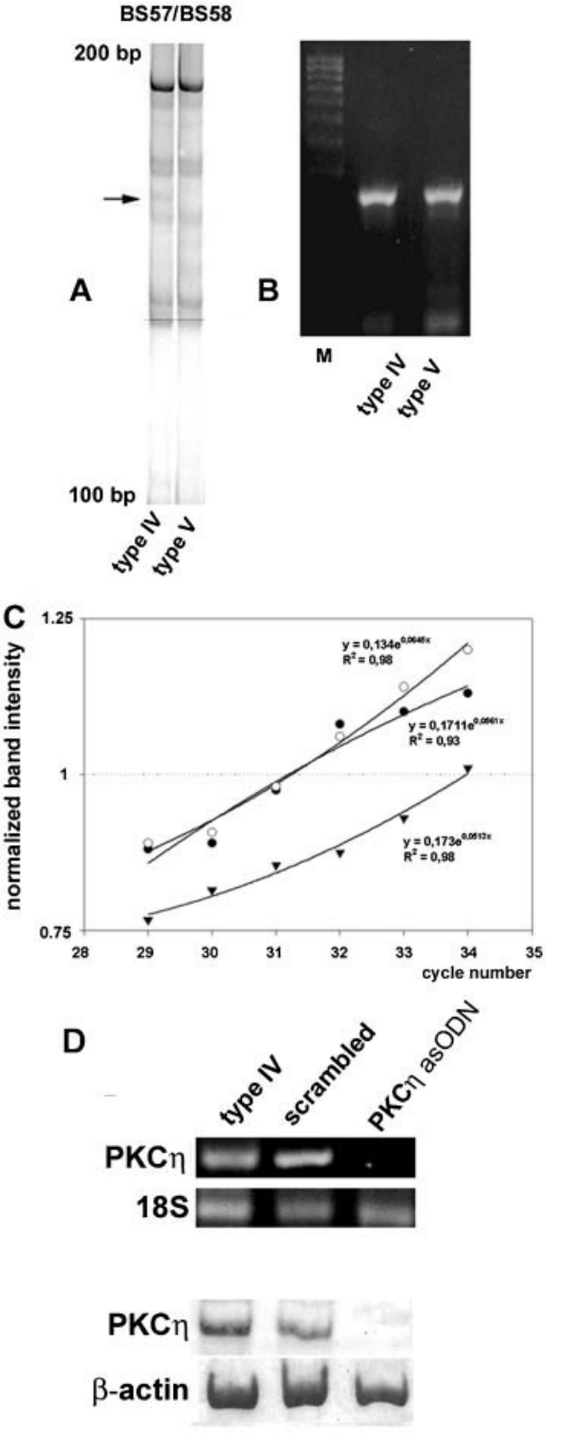

Figure 1. Identification of PKCη as a differentially-expressed gene and effect of transfection on PKCη expression. A: DD-PCR of cDNA preparations from 8701-BC cells cultured onto type IV and V collagen substrata. The arrows point to a band of about 160 bp, selectively displayed in the type IV collagen-derived sample. Detail of a 6% sequencing PAGel, silver staining. B: Conventional PCR amplification of the product of *PKCη* cDNA in cells cultured onto type IV and V collagen substrata. Cycle number was 35. Agarose gel 2%, ethidium bromide stain. *M* = 100 bp size marker. C: SM-PCR for *PKCη*. Representative plot of normalized data versus cycle numbers fit with an exponential curve for cells grown onto type V (▼), control type IV collagen (●) and control tissue culture plastic (○). D: (Top) PCR amplification of the product of *PKCη* cDNA in cells cultured onto type IV collagen substrata, and transfected with ''scrambled'' asODN or PKCη asODN. Cycle number was 35. 18S = bands of the ''housekeeping'' gene after 10 cycles of PCR amplification to check the quality and the content of the three cDNA samples. (Bottom) Western immunodetection of PKCη in preparations from cells cultured in the same experimental conditions as above. Immunoreaction with anti-β actin antibody was performed to check the quality of the lysate and protein loading.

conditions, indicating that expression of *PKCη* was switched-on in the presence of both type IV and V collagen substrata. For semi-quantitative evaluation three cDNA preparations (from cells cultured on type IV- or type V collagen and on tissue culture plastic as a further control) were submitted to SM-PCR as described. As shown in Figure 1C, growth of 8701-BC cells onto type V collagen substrate resulted in the down-regulation of *PKCη* of about 6.5-fold with respect to cells plated onto either type IV collagen or plastic.

Antisense-Mediated Down-Regulation of PKCη in 8701-BC Cells Grown Onto Type IV Collagen Affects Cell Proliferation and Morphological Appearance

In order to determine whether the decrease of *PKCη* expression levels might be in some way implicated in the already-reported modifications of cell morphology, growth rate and expression levels of apoptosis-related genes [13,14,16], in a second set of assays we induced asODN-mediated down-regulation of *PKCη* in 8701-BC cells grown onto control type IV collagen substrate and performed microscopic observations and cell number evaluations in parallel with non-transfected and ''scrambled'' asODN-transfected cells grown onto the same substrate, and non-transfected cells grown onto type V collagen substrate.

Preliminarily, we checked at mRNA and protein level whether exposure to the specific asODN was successful in down-regulating *PKCη*, through PCR amplification of preparations of cDNAs and immunodetection on protein extracts from parallel samples. As shown in Figure 1D, incubation with lipofectin-vehiculated PKCη-asODN resulted in the absence of both amplification of the cDNA fragment and immunorevelation of the protein product, at least under the detection conditions used.

Images of randomly-selected microscopic fields containing sparse 8701-BC cells grown onto the different collagen substrata, with or without asODN-transfection, were recorded and analyzed. The results in Figure 2A show that after 48 h of culture untreated or ''scrambled'' asODN-treated cells seeded onto type IV collagen appeared to be well-spread on the substrate displaying an assortment of morphological appearances with many elongated fibroblastoid and scattered polygonal/polymorphic giant elements. Conversely, PKCη asODN-treated cells plated onto type IV collagen closely resembled 8701-BC cells grown onto type V collagen substrate, displaying a less-elongated morphology with several elements loosely-attached to the substrate or rounded, as is typical for unhealthy cells. Cell number was measured 48 h after plating onto either substrate, with or without transfection. In line with the microscopic observations, the number of PKCη asODN-treated 8701-BC cells onto type IV collagen was comparable to that of untreated cells grown onto type V collagen, both being much lesser that those of control and ''scrambled'' asODN-treated cells (Figure 2B). On the other hand, the number of cells grown onto control tissue culture plastic was comparable to that of untreated cells grown onto type IV collagen substrate (not shown).

Antisense-Mediated Down-Regulation of PKCη in 8701-BC Cells Grown Onto Type IV Collagen Induces DNA Fragmentation and Affects the Expression Levels of Apoptosis-Related Genes

We therefore examined whether the down-regulation of *PKCη* experimentally-induced in 8701-BC cells grown onto type IV collagen substrate might result in apoptosis-related DNA fragmentation and changes of gene expression levels, similar to those reported in a previous publication [16]. First, cells cultured onto type IV collagen, untreated or transfected with either PKCη asODN or ''scrambled'' asODN, were stained following the ApopTag protocol and examined under the microscope. As shown in Figure 3, PKCη asODN-transfected cells stained positively for apoptosis using the apoptosis-discriminating ApopTag oligo A.

Then, we submitted cDNA preparations obtained from cells grown in each experimental condition to conventional PCR to check whether the selected genes were expressed. As shown in Figure 4A, amplification bands for *Bcl-2*, *Bcl-Xs*, *Bad*, and *Dap kinase* (*Dapk*) could be observed in the preparations obtained from cells grown onto type IV collagen, both control and transfected. The amplification product for *caspase 5* was found only in cDNA samples from *PKCη* asODN-treated cells. When 35 cycles of amplification were performed to detect the product of *caspase 8*, a band of remarkable intensity was found in preparations from *PKCη* asODN-treated cells, being consistently weaker after the amplification of the other two cDNA samples.

To check whether the expression levels of *Bcl-2*, *Bcl-Xs*, *Bad*, and *Dapk* were differing among the three experimental conditions, we then submitted the cDNA preparations to SM-PCR. As shown in Figure 4B and C, when compared to non-transfected control, exposure to ''scrambled'' asODN did not alter the expression levels of the genes under study, whereas transfection with PKCη asODN resulted in the up-regulation of *Bcl-Xs* (approx. 2-fold), *Bad* (approx. 3-fold) and *Dapk* (approx. 2.5 fold), and in the down-regulation of *Bcl-2* (approx. 2-fold). Table 1 reports a synopsis of the expression level ratios of the latter genes in untreated 8701-BC cells cultured onto type V collagen [16] and in PKCη asODN-transfected cells cultured onto type IV collagen.

The same PCR and SM-PCR assays were also performed for the other caspase-encoding genes,

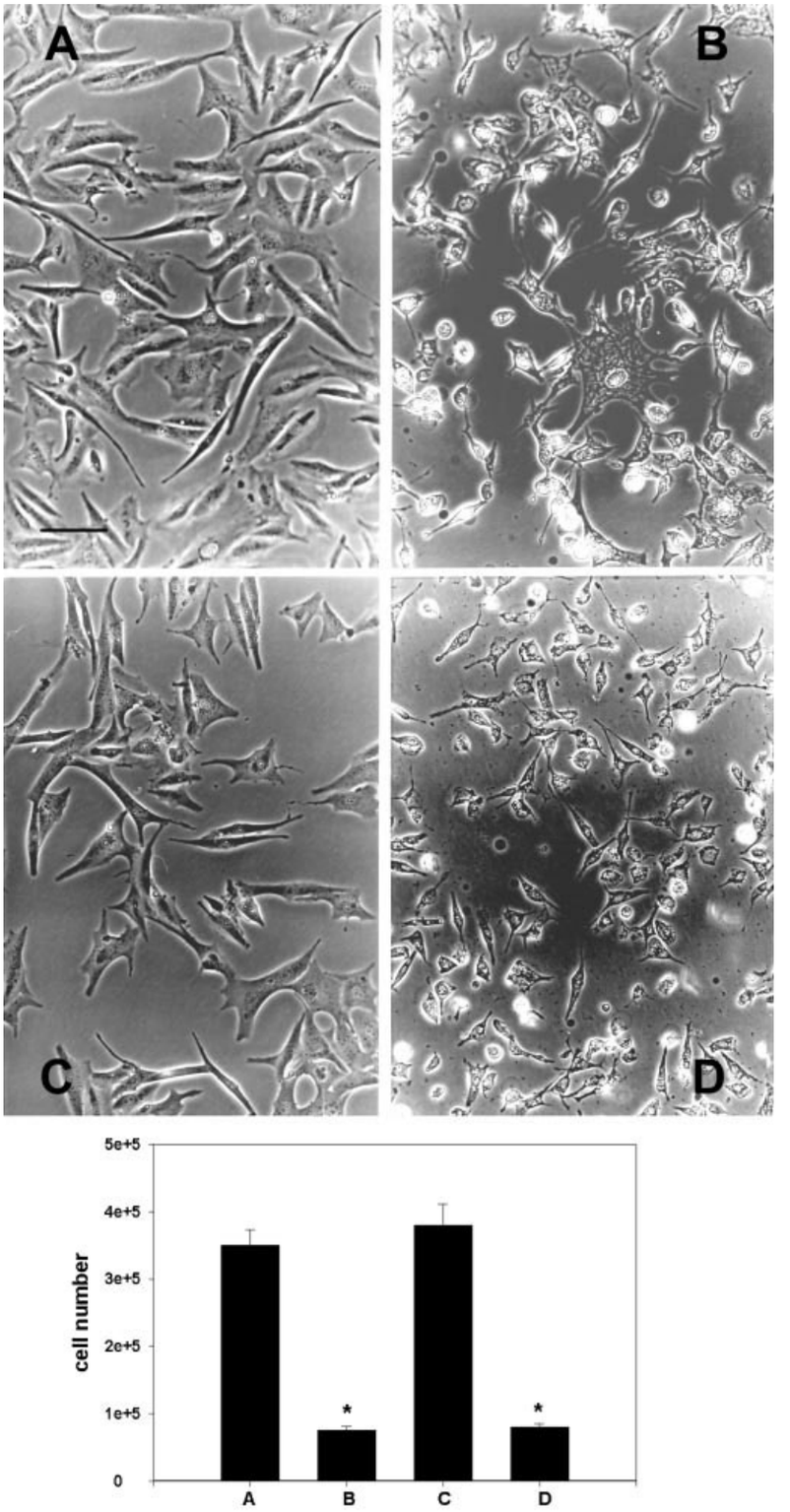

Figure 2. Effect of transfection on cell morphology and number. (Top) Representative phase contrast micrographs of 8701-BC cells grown for 48 h onto either type IV (A,B,C) or type V collagen substrate (D) and transfected with PKCη asODN (B) or ''scrambled'' asODN (C). Bar = 10 μm. (Bottom) Histogram showing the number of 8701-BC cells in the same experimental conditions as above. Data are from quadruplicate experiments. $^*P < 0.05$ with respect to control sample (non-transfected cells cultured onto type IV collagen).

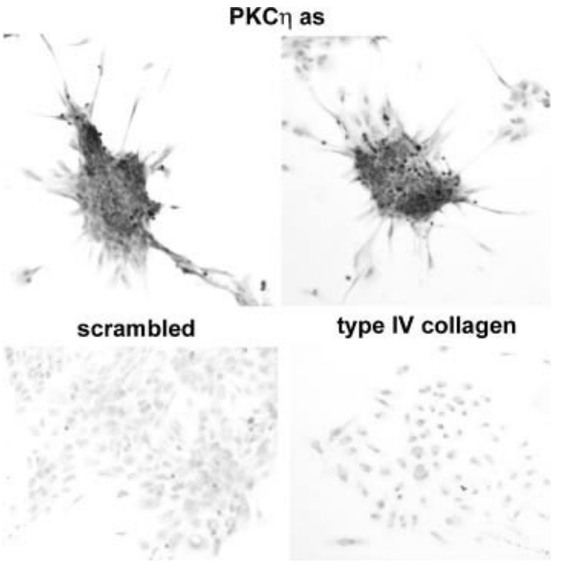

Figure 3. Effect of transfection on DNA fragmentation. Panel of micrographs of 8701-BC cells cultured onto type IV collagen, untreated or transfected with either PKCη asODN or ''scrambled'' asODN, and submitted to ApopTag labelling using apoptosis-discriminating oligo A (one-base dT overhang-end detection).

whose level of expression was found modified following culture onto type V collagen by Luparello and Sirchia [16], i.e., *caspase 1*, *9* and *14*, but no significant difference was found between PKCη asODN-treated cells and controls grown onto type IV collagen (data not shown).

DISCUSSION

It is widely-acknowledged that the PKC family of serine/threonine kinases includes the subfamilies of conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ) and atypical isoforms (ζ, λ, and ι) which differ for their structural properties and sensitivity to the cofactors DAG and Ca^{++} [27], and that several members of this family exert critical effects on the control of cell survival, growth, differentiation, motility and death [28]. PKCη, phospholipid-dependent and Ca^{++}-independent enzyme, is predominantly expressed in diverse epithelial tissues, such as skin, tongue, forestomach, intestine and mammary gland alveoli, and its expression was also found associated to the progression of renal and breast carcinomas [29–32].

A number of data, on the other hand, have brought evidence for a role of PKCη as an actual inhibitor of the apoptotic process. For example, Akkaraju and Basu [33] have demonstrated that *PKCη* over-expression in MCF-7 breast cancer cells is able to attenuate TNF-induced caspase 7 and 8 activation and cell death, whereas Hussaini et al. [34] reported the PKCη-controlled block of the apoptotic cascade by preventing caspase 9 activation in glioblastoma cells submitted to UV and γ radiations, thereby indicating the enzyme as an interesting target for chemotherapeutical intervention aimed to control the evolution of this neoplastic histotype [35]. Moreover, PKCη was proven to be effective in mediating the proliferative response to phorbol esters, and blocking the onset of apoptosis by preventing caspase 3 activation also in irradiated human keratinocytes [36]. Concerning the molecular pathways associated to the activation of the enzyme, although data are still fragmentary, it is known that it functions as a powerful activator of Raf1, protein kinase involved in drug resistance [37], and that it associates with cyclin E/cdk2/p21 complex, thereby phosphorylating p21 and inhibiting cdk2 kinase in keratinocytes [38]. In MCF-7 breast cancer cells, the anti-apoptotic role of PKCη was partly explained in light of the observed up-regulation of G_1 phase cyclins D and E, and suppression of c-Jun N-terminal kinase (JNK) activity, the latter being a dissimilarity to most PKC isoforms [39,40].

Dealing with mammary gland tissue, interestingly Masso-Welch et al. [30] reported that in the rat *PKCη* undergoes to up-regulation following gland activation during pregnancy and consequent growth and differentiation of the alveolar epithelium, whereas its protein level decreases drastically during physiological involution, an aspect that can be related to the promotion of apoptotic events typical of gland regression. A positive effect of PKCη on breast epithelial cell survival and proliferation was also observed in tumor model systems by Karp et al. [41] who reported its up-regulation in estrogen-responsive MCF-7 and T47-D human cancer cells treated with 17 β-estradiol in a time and dose-dependent manner.

Here we have demonstrated that 8701-BC cells cultured onto type V collagen undergo to apoptosis-oriented phenotypical modifications, including changes in gene expression levels which appear to be, at least in part, downstream to *PKCη* down-regulation. Further studies will be required to determine the precise cellular pathways that lie beneath the suggested interactions; however, the data we present allow the following comments.

Present results expand the list of human neoplastic cell lines whose life/death decisions contemplate the involvement of PKC*η* and also further support the concept of an anti-apoptotic role played by the kinase, already suggested for other model systems (e.g., [23,34,36,40,42]). In addition, to our knowledge they report the first evidence of the modulation of *PKCη* expression by an endogenous ECM component, type V collagen, physiologically-present in breast stroma in low amount and increased in cases of d.i.c. as a putative ''cancer-restraining'' reaction of the host tissue

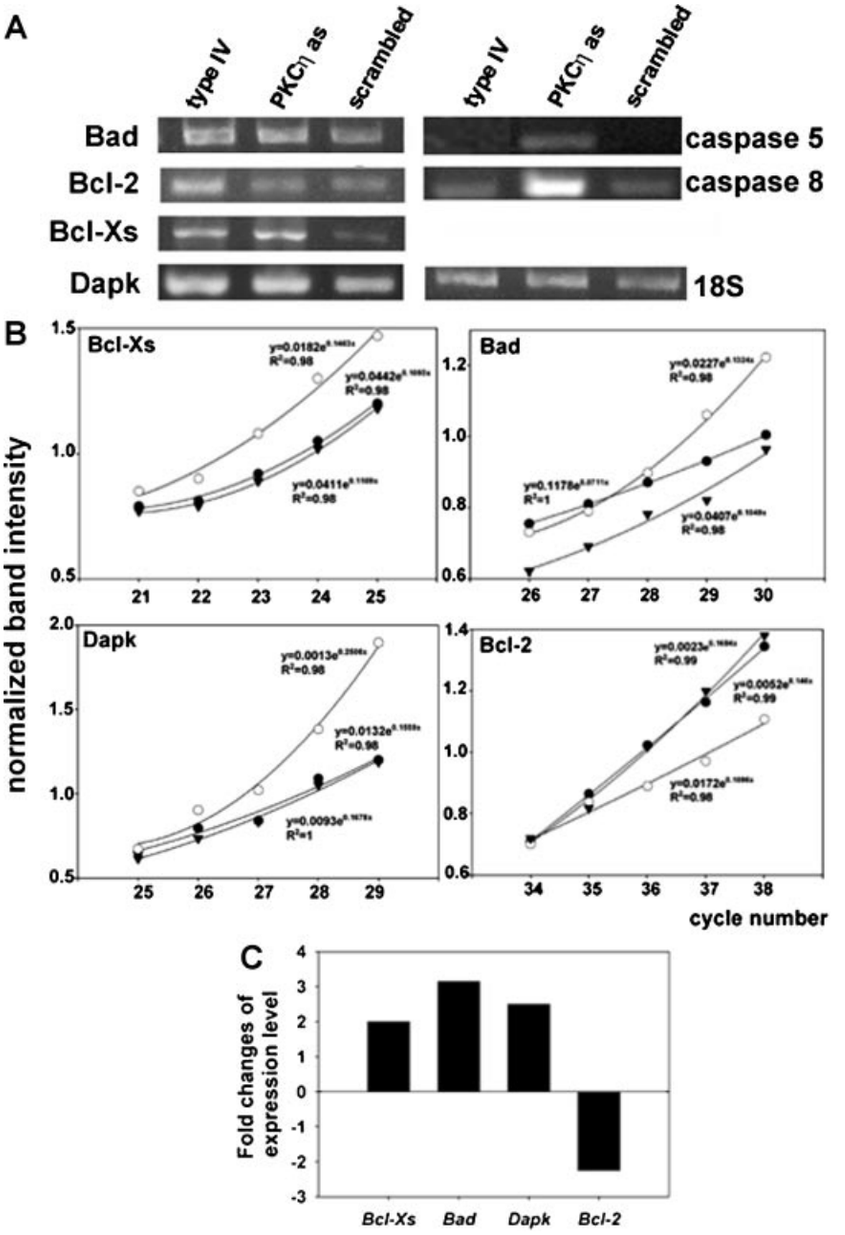

Figure 4. A: Effect of transfection on gene expressions. The image shows the bands obtained from preparations of cells cultured onto type IV collagen substrata, and transfected with either PKCη asODN or ''scrambled'' asODN. PCR cycle number was 35. 18S = bands of the ''housekeeping'' gene after 10 cycles of PCR amplification to check the quality and the content of the three cDNA samples. B: SM-PCR for *Bcl-Xs*, *Bad*, *Dapk*, and *Bcl-2*. Representative plots of normalized data versus cycle numbers fit with an exponential curve for cells grown onto type IV collagen, untreated (●) or transfected with either PKCη asODN (○) or ''scrambled'' asODN (▼). C: Histogram showing the average changes in expression levels of the four genes tested based on SM-PCR assays of PKCη asODN-treated 8701-BC cells grown onto type IV collagen substrate compared with untreated controls (SEM was less than 5% in each experimental condition).

[43,44]. In the absence of the ''less permissive'' type V collagen substrate, cells cultured onto the more ''adequate'' type IV collagen substrate and submitted to the antisense-mediated experimentally-induced down-regulation of *PKCη* display the same changes in cell morphology, proliferative activity, DNA fragmentation and gene expression levels, thereby strongly indicating the central role of the kinase in orchestrating cell death program. Apart from the well-known anti-apoptotic gene *Bcl-2*, the other genes taken into account were the pro-apoptotic *Bcl-X*, *Bad* and *Dapk*. The first two

Table 1. Expression Level Ratio of Apoptosis-Related Genes in 8701-BC Cells Cultured onto Either Type V Collagen (V) or Type IV Collagen in the Presence of PKCη asODN (IV + asODN)

Gene	V	IV ± asODN
Bcl-2	1:2.6	1:2.25
Bcl-Xs	2.8:1	2:1
Bad	1.9:1	3.15:1
Dapk	2:1	2.5:1

are members of Bcl-2 related protein family. Bcl-xS is the short (s) splice variant of Bcl-X, encoding for a protein of 170 aminoacids which antagonizes the cell-survival function of the longer variant Bcl-xL; the ability of Bad to bind to anti-apoptotic members of the same family has also been recognized [45,46]. Dapk is a Ca^{2+}/calmodulin-regulated Ser/Thr kinase endowed with cell death-associated functions; Extracellular signal-regulated kinase (ERK) and UNC5H2-dependence receptor are known to associate to the enzyme and to regulate its activity [47]. Dapk localizes to actin microfilaments and can induce focal adhesion disassembly with consequent perturbation of the balance between contractile and adhesion forces and subsequent cell detachment [48]; it is therefore conceivable that PKCη-dependent up-regulation of *Dapk* might contribute to the morphological changes and prominent decrease in cell spreading observable when 8701-BC cells are seeded onto type V collagen substrate. More recently, a number of experimental data have provided evidence of a link between Dapk and promotion of autophagy as a response to endoplasmic reticulum stress [49]. However, whether type V collagen-triggered *Dapk* up-regulation via PKCη may lead to the enhanced phosphorylation of beclin-1, being one of the substrata of the enzyme, thereby initiating the autophagic process, remains still to be determined. Concerning our results on caspases, although examining expression at the mRNA level could provide evidence of differences that do not necessarily correspond to changes in enzyme activity, nonetheless preliminary results have suggested the enhanced degradative activity of lysates from PKCη asODN-treated cells *vs.* controls, whose quantitative evaluation awaits further investigation (Luparello, unpublished observations). On the other hand, our results give an indication of which caspases may be regulated during incubation of 8701-BC cells with PKCη asODN. The absence of modulation of *caspase 1*, *9* and *14* expression in the present experimental system indicates that they are under the control of mechanisms distinct from PKCη down-regulation, whose nature is still to be determined.

In conclusion, our data provide additional information on the intracellular pathways that are triggered by 8701-BC breast cancer cell-type V collagen interactions. Taking present and previous results produced by our laboratory together [13–17], they collectively represent the first documented indication, to our knowledge, that an endogenous tissue component, like type V collagen, is able to promote apoptosis-like death in breast cancer cells. Interestingly, we have produced evidence that also T47-D breast cancer cells are responsive to the death-promoting effect of type V collagen [21], thus supporting the putative ''anticancer'' role of type V collagen on cells of the d.i.c. histotype irrespective of their source (primary site for 8701-BC cells and pleural effusion for T47-D cells). Besides, preliminary results indicate that *PKCη* down-regulation occurs also in T47-D breast cancer cells cultured onto type V collagen substrate, although to a lesser extent (Luparello, unpubl. observations), thereby further suggesting the generality of the observed process and prompting to utilize both cell lines to break down and analyze in detail the signaling pathways involved in the modulation of *PKCη* expression.

In addition, the present data further substantiate our previous postulates that in cases of breast d.i.c. the zonal increase of type V collagen contribute to the assembly of a ''non-permissive'' micro-environment for tumor cells, antagonist to other local permissive substrata ([4] for review). It is therefore conceivable that the dynamic turnover of ''instructive'' stromal components in the affected breast may actively intervene in the modulation of neoplastic cell behavior and metastatic propensity, thus directing the tumor cell population towards different levels of malignancy.

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