Type V collagen induces apoptosis of 8701-BC breast cancer cells and enhances m-calpain expression

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Abstract

Introduction

We previously reported that ductal infiltrating carcinomas (DIC) of the human breast display profound modifications of the stromal architecture, associated with anomalous collagen composition. The major alterations observed in the interstitial collagen were an abnormal ratio between type I and type III collagens, the appearence of an onco-foetal form of collagen (OF/LB) and a relative increase of type V collagen content. Biological assays performed by culturing a DIC-derived cell line (8701-BC) onto type V collagen substrate demonstrated that the latter was able to restrain cell growth and to inhibit cell motility and invasion "in vitro", differently from what found with other collagen species tested.

Aims

To search for molecular mechanisms underlying the observed inhibitory effect of collagen type V on breast cancer cells. As a reference model, we used culture substrates prepared with type IV collagen, which represents the physiological substrate for cells of epithelial origin.

Methods

Apoptosis was studied by both fluorescence microscopical analyses of cell viability and DNA fragmentation assays in preparations of 8701-BC cells grown onto either type V and type IV collagen. Differences in gene expression following cell adhesion onto the two substrates were analyzed by a "differential display" PCR (DD-PCR) technique and Western blot.

Results

   In this paper we demonstrate that the inhibitory effect exerted by type V collagen is consistently associated with the switching-on of a death program by a significant proportion of the cell culture, concomitant with the formation of cohesive cell islands displaying a progressive decrease of cell spreading. DD-PCR and Western blot assays demonstrated a consistent association of type V collagen-promoted apoptosis with the up-regulation of the large subunit of m-calpain (L-mC) at both mRNA and protein level. Cell exposure to calpain inibitor I decreased the amount of DNA fragmentation by 30%.

Discussion

   The present data substantiate our previous postulates that in cases of breast DIC 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 substrates. It is therefore conceivable that the spatio-temporal derangement of stromal components may actively modulate neoplastic cell behavior and metastatic propensity, thus contributing to the selection and development of more or less malignant tumor phenotypes.

Keywords

INTRODUCTION

   The interdynamic space-temporal interactions of given cells with their extracellular matrix (ECM) are known to drive morphogenesis during development and to maintain the normal tissue architecture and functions in adult organs. Cellular behaviors that are modulated by (or associated to) cell-matrix interactions include growth [1,2], differentiation [3,4,5], apoptosis [6], motility [7,8,9], signal transduction and gene expression [10,11,12,13,14], to quote among the major effects. Local disruption of ECM after pathologic events, may result in selective reprogrammed cell behavior, through a cascade of signals difficult to predict *a priori*.

   Some previous data obtained in one of our laboratories have shown that in cases of the ductal infiltrating carcinoma (DIC), a highly malignant tumor of the human breast, the stromal ECM undergoes to extensive regional remodelling with enhanced deposition of collagen components, mostly modified with respect to the normal counterpart [15,16]. The major aspects were the over-deposition of type V collagen normally expressed at very low levels in adult tissues, and the appearence an onco-foetal form of interstitial collagen (OF/LB) [17,18,19]. Biological assays performed with a neoplastic cell line

(8701-BC), isolated from a primary DIC [20], demonstrated that different collagen substrates were able to promote distinct behaviors of the cell population: in particular, the onco-foetal collagen enhanced cell proliferation and migration and promoted invasivity both "in vitro" and "in vivo", whilst type V collagen exerted an antagonistic effect, restraining both cell growth and "in vitro" invasivity [2,8,21,22,23,24].

In consideration of the potentially-relevant implications of restrictive forces on tumor progression, we set out experiments to understand how this inhibitory effect of collagen type V is exerted on cancer cells. As a reference model, we used culture substrates prepared with type IV collagen, which represents the physiological substrate for cells of epithelial origin. First, to address the question whether type V collagen was able to promote programmed cell death, we performed fluorescence microscopical analyses of cell viability and DNA fragmentation assays, both by gel electrophoresis and centrifugal sedimentation. Secondly, we investigated the possibility of some transcriptional specificity induced by type V collagen substrates on 8701-BC cells, by applying the technique of the "differential display"-polymerase-chain-reaction (DD-PCR) described by Sokolov and Prockop [25] 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.

By this experimental approach, here we demonstrate that type V collagen is able to promote apoptosis in a large fraction of 8701-BC cells, and that the apoptotic process is associated with a marked increase of expression and production of the large subunit of m-calpain (L-mC; EC 3.4.22.17), a neutral Ca^{++}-dependent non-lysosomal enzyme belonging to the cystein protease family ([26,27,28] for reviews).

MATERIALS AND METHODS

Cell cultures

8701-BC cells were routinely cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and antibiotics. Collagen type IV and V were purchased from Sigma (St.Louis, MO/USA). Collagens were dissolved in 0.5 M acetic acid, sterilized with chloroform as already reported in Luparello *et al.* [2], plated in 25 cm^2 flasks at the concentration of 10 μg/cm^2 for 48 h and exhaustively neutralized with PBS just before cell culturing. 8701-BC cells, detached with 0.1% EDTA, were plated in substrate-coated flasks at the concentration of 3.5 x 10^4/cm^{2}in serum-free conditions and allowed to grow for 48 h.

Fluorescence microscopy

A mixture of acridine orange and ethidium bromide (both purchased from Sigma and dissolved at 2 μg/ml of PBS) was added to the culture medium of unfixed 8701-BC cells grown for 48 h onto either

type IV or type V collagen substrate, and the cells viewed immediately under either FITC (for acridine orange) or TRITC (for ethidium bromide) fluorescence [29] and photographed.

DNA fragmentation assays

The occurrence of DNA fragmentation was evaluated electrophoretically as described by Navarro *et al*. [30] with slight modifications, and its extent checked by centrifugal sedimentation as reported by Duke and Cohen [31].

For the electrophoretic assay, 8701-BC cells were plated onto either type IV collagen- or type V collagen-coated 6-well dishes, at the concentration of 6×10^5 (for type IV collagen) and 1.2×10^6 cells/well (for type V collagen) and grown for 48 h in serum-free medium. At the end of the incubation, cells were scraped from the plates, lysed in 100 μl of 45 mM Tris-borate, pH 8, containing 0.25% Nonidet P-40 substitute and 10 mM EDTA, and treated for 1 h at 50°C with 1 mg RNase A/ml (Fluka, Buchs/CH) and then for 1 h at 50°C with 1 mg Proteinase K/ml (Fluka). Samples were mixed with loading buffer (10 mM EDTA, pH 8, plus 0.25% bromophenol blue, 40% sucrose and 1% low melting point-agarose) and, in order to improve band resolution, they were submitted to voltage gradient gel electrophoresis (VGGE; [32,33]) in ethidium bromide-containing 1.2% agarose gel with TAE buffer for 5 h at 60 V.

For the quantitative evaluation of DNA fragmentation, 8701-BC cells were plated at the concentration of 1×10^5 cells/well onto either type IV collagen- or type V collagen-coated 4-well dishes in serum-free conditions and allowed to adhere onto the substrates for 24 h. Subsequently, a set of cells was incubated with 2 μCi/ml [methyl-^{3}H] thymidine ([^{3}H]TdR), for 18 h, whilst in parallel preparations, the 18 h-treatment with [^{3}H]TdR was performed after additonal 24 h (i.e. at 48 h from seeding). Cells were then exhaustively washed with RPMI 1640 medium pre-warmed to 37°C and lysed with TTE solution (10 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100 and 1 mM EDTA); the fragmented DNA was then separated from intact chromatin by centrifugation. The radioactivity present in both the supernatant and the pellet, the latter resuspended in 1% SDS, was evaluated by liquid scintillation counting. Percent DNA fragmentation was calculated as [(c.p.m in the supernatant)/(c.p.m. in the supernatant plus c.p.m. in the pellet)]. Data are presented as mean ± s.e.m. 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 samples and controls. the dna fragmentation assay following calpain inhibition followed essentially the same protocol with the exception that either 20 μm calpain inhibitor i (N-acetyl-Leu-Leu-norleucinal purchased by Boehringer; [34]) in DMSO or DMSO vector only were added to cell plating medium.

Messenger RNA extraction and reverse transcription

Isolation of polyA$^+$ mRNA from monolayers of 8701-BC cells grown for 48 h onto either type IV or V collagen substrate was carried out with oligo-dT$_{25}$tailed magnetic beads using the mRNA DIRECT kit purchased from Dynal (Oslo/N). The protocol for the latter included the preparation of crude cell lysate, annealing of polyadenylated mRNA to the beads, washing of the suspension and elution of purified transcripts. Samples of 0.1-0.5 μg of mRNA were submitted to cDNA synthesis in the presence of random 6-mer primers, using the SuperScript Preamplification System (Gibco), according to manufacturer's instructions.

Differential Display-Polymerase Chain Reaction (DD-PCR) and Sequencing

For differential expression analysis, DD-PCR experiments were performed using the arbitrary 10-mer primers designed by Sokolov and Prockop [25], i.e. BS52 (5'-CAAGCGAGGT-3'), BS54 (5'-AACGCGCAAC-3'), BS55 (5'-GTGGAAGCGT-3') and BS57 (5'-GGAAGCAGCT-3'), in combinations of two. The PCR amplification was carried out using 25 pmoles of each of two primers, 1-2 μl of the cDNA template and 3.6 U of AmpliTaq DNA Polymerase, Stoffel fragment (Perkin Elmer, USA), 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. PCR products were analysed by 6% non-denaturing polyacrylamide gel electrophoresis, followed by silver staining [35,36]. PBR322 plasmid digested with *Hinf*I was used as size marker (154, 220/221, 298, 344, 396, 506, 517 and 1,631 bp fragments). The cDNA fragment of interest was excised from the gel and subjected to several repetitions of amplification and electrophoresis until purity. Direct dideoxy-sequencing of both strands of the PCR product, purified with the High Pure PCR Product Purification Kit (Boehringer), was carried out with Sequenase (Amersham, UK) in the presence of either BS52 or BS57 primer and ^{33}P-labelled dATP. DNA sequence similarity was searched with the BLAST algorithm [37] available on-line at http:\\www.ncbi.nlm.nih.gov.

Semi-quantitative PCR

Non-competitive semi-quantitative PCR was performed at low cycle number [38] using 50 μM of the primers for L-mC (accession nr. M23254), i.e. 5'-CAAAAACTTCTTCCTGACGAATCG-3' (sense) and 5'-CCAGACCTGTCAACGTCGATT-3' (antisense), designed with the Primer Selection software available on-line at http://http:\\alces.med.umn.edu, and specific for a 512 bp sequence from bases 1477-1989 of the L-mC coding sequence [39]. Cycle profile was 94°C for 2 min., followed by 18 cycles of 94°C for 30 sec., 47°C for 45 sec., 72°C for 1 min (5 min. during the last cycle). Primewax (Biometra) was added for "hot start". GAPDH was amplified in parallel as described by Southby *et al*. [40]. PCR products and size marker (PBR322 plasmid/*Hinf*I) were analysed by 6% PAGE and visualized at 254 nm transillumination after staining with SYBR Green I. SigmaGel 1.0 software was utilized for the evaluation of pixel intensity of the fluorescent bands. A diagnostic restriction test was designed using the WWW tacg program, available at http:\\hornet.bio.uci.edu; according to the data

obtained, the amplification product of L-mC cDNA, purified by High Pure PCR Product Purification Kit (Boehringer), was digested with excess *Pst*I enzyme and the digest analyzed by PAGE as described.

# Protein extraction and Western blot

Cells were plated in collagen-coated flasks in serum-free conditions and allowed to grow for 48 h, as already reported. Cell lysis was performed with 20 mM Tris-HCl, pH 7.4, containing 2% SDS, 5 mM EGTA, 5 mM EDTA, 0.5 mM PMSF, 5 µg leupeptin/ml and 10 µg aprotinin/ml; total proteins were extracted and TCA precipitated according to Wang *et al*. [41]. Measured aliquots of the extracted proteins were subjected to SDS-PAGE (7.5% acrylamide). Western-blot was performed with anti-L-mC antibody from rabbit (1:1,000; Swant, Bellinzona/CH), and the reaction revealed by horseradish peroxidase-conjugated anti-rabbit IgG (1 U/ml; Boehringer) and staining with diaminobenzidine according to [34]. The evaluation of the pixel intensity of the bands was done by automated scanning using SigmaGel 1.0 software. Beta-actin was immunostained in parallel to confirm the loading of the proper amount of material.

RESULTS

Apoptosis of 8701-BC cells cultured onto type V collagen

For the microscopical investigation of cell viability we performed double-fluorescence assays, after staining unfixed cells with acridine orange and ethidium bromide, being the latter unable to permeate the intact membrane of live cells and thereby representing a marker for morphological detection of cell death. As shown in fig. 1, panel A1-3 (FITC filter for acridine orange), cells plated onto type IV collagen reached confluence 48 h after seeding, acquiring a spread morphology and organizing themselves in a uniform and continuous monolayer covering the total culture area. Conversely, cells plated onto type V collagen (fig. 1, panel B1-3) grew as cohesive islands of different size, with increasing cell-cell contacts and progressive decreasing of cell extensions. Shifting to the TRITC filter, the same fields were observed for ethidium bromide staining: as shown in fig. 1, panel A 4-6, cell plated onto type IV collagen exhibited a very limited uptake of ethidium bromide, while cells attached onto type V collagen (fig. 1, panel B 4-6) displayed a more marked level of cell death.

Figure 1 Fluorescence microscopical analysis of 8701-BC cells grown for 48 h onto either type IV (panel A) or type V collagen substrates (panel B) and stained with acridine orange (1-3) and ethidium bromide (4-6). Microscopical magnification 40 X.
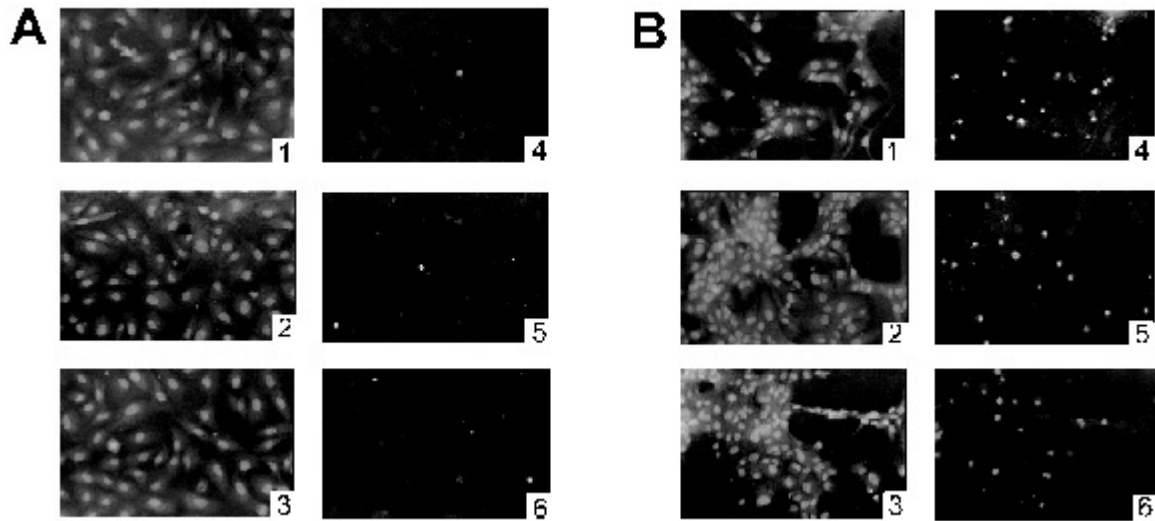
In light of these morphological observations, we then performed an electrophoretic assay to check the status of the DNA extracted from cells cultured onto type IV and type V collagens. The DNA ladder, indicative of internucleosomal DNA fragmentation, was apparent after 48 h of growth onto type V collagen substrate, whilst only intact high-molecular weight DNA was present in cells grown onto type IV collagen substrate (fig. 2). The amount of fragmented DNA in cells cultured onto either substrates was therefore quantitated by centrifugal sedimentation after labelling with tritiated thymidine, at 48 and 72 h after plating (fig. 3). As expected, cells cultured onto type V collagen substrate, had a much higher level of DNA fragmentation, both at 48 and 72 h after seeding ($86.1 \pm 1.1\%$ and $87.25 \pm 0.35\%$, respectively) compared to that of cells cultured onto type IV collagen ($16 \pm 0.01\%$ and $13.25 \pm 1.25\%$, respectively).

Figure 2 VGGE of the DNA isolated from 8701-BC cells grown for 48 h onto either type IV or type V collagen substrates. Agarose 1.2%, ethidium bromide stain

Figure 3 Extent of DNA fragmentation in 8701-BC cells after 48 and 72 h from seeding onto either type IV or type V collagen substrates. Vertical bars indicate the s.e.m. of quadruplicate experiments. *$p < 0.05$ (type V versus type IV collagen)
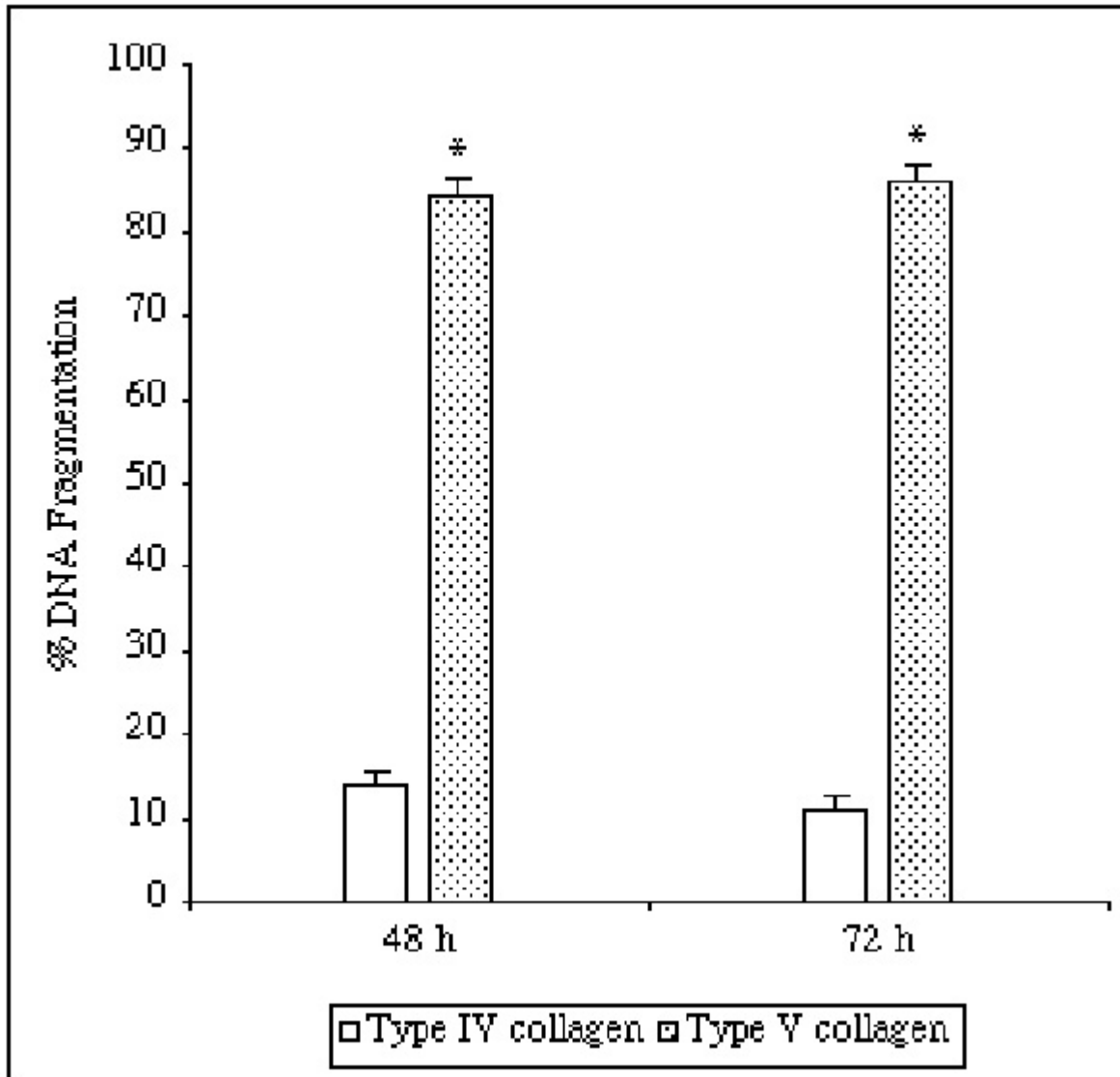

Up-regulation of L-mC in 8701-BC cells cultured onto type V collagen

In order to search for putative molecular markers linked to the apoptotic switch induced by type V collagen, we submitted cDNA preparations, obtained from 8701-BC cells grown onto the two different substrates, to DD-PCR as described. Only one out of the four combinations of arbitrary primers utilized (i.e. BS52-BS57) gave an amplification band of approx. 165 bp selectively present in the cDNA preparation of cells cultured onto type V collagen (fig. 4). The 165 bp-cDNA fragment was purified by excision from the gel and several cycles of PCR and electrophoresis and submitted to direct dideoxy-sequencing of both strands; homology was found in the EMBL and GeneBank DNA data bases between

the partial sequence obtained and that of region 2148-2225 of the coding sequence for *L-mC* (accession number M23254) as deposited by Imajoh *et al*. [39].
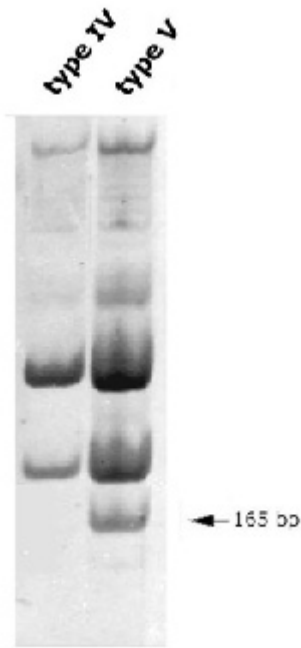
To check the differential expression of *L-mC* under alternative experimental conditions enhancing the stringency and sensitivity of the test, a new set of cDNA samples from 8701-BC cells was subjected to PCR analysis in the presence of two primers specific for L-mC mRNA, designed by the "Primer selection" software available on-line. As shown in fig. 5A, the PCR amplification gave a product which displayed a size (512 bp) identical to that expected theoretically; moreover, to confirm its identity, the amplification product was submitted to diagnostic restriction analysis with *Pst*I, successfully releasing the two fragments of 196 and 316 bp predicted. For semi-quantitative evaluation, the various cDNA preparations from 8701-BC cells were submitted to PCR analysis keeping cycle number low to maintain the linear relationship between input RNA and final product [38] and amplifying the "housekeeping" gene *GAPDH* in parallel as a control for starting material. The little amount of PCR product generated was revealed by the highly-sensitive SYBR Green I stain. As shown in fig. 5B, when PCR amplification was performed in the presence of the specific primers, the signal for L-mC (normalized to that of GAPDH) was at least four times higher in cells plated onto type V collagen than in cells grown onto type IV collagen.

Figure 5 A) PCR amplification of the 512 bp product of L-mC cDNA (1), and diagnostic restriction analysis of the same yielding the expected 316 and 196 bp fragments after digestion with *Pst*I endonuclease (2). M) Size marker PBR322 plasmid/*Hinf*I. B) "Low cycle number"-PCR amplification of cDNA preparations from 8701-BC cells grown onto either type IV or type V collagen substrates in the presence of primers specific for L-mC and GAPDH cDNA fragments. PAGE 6%, SYBR Green I stain.
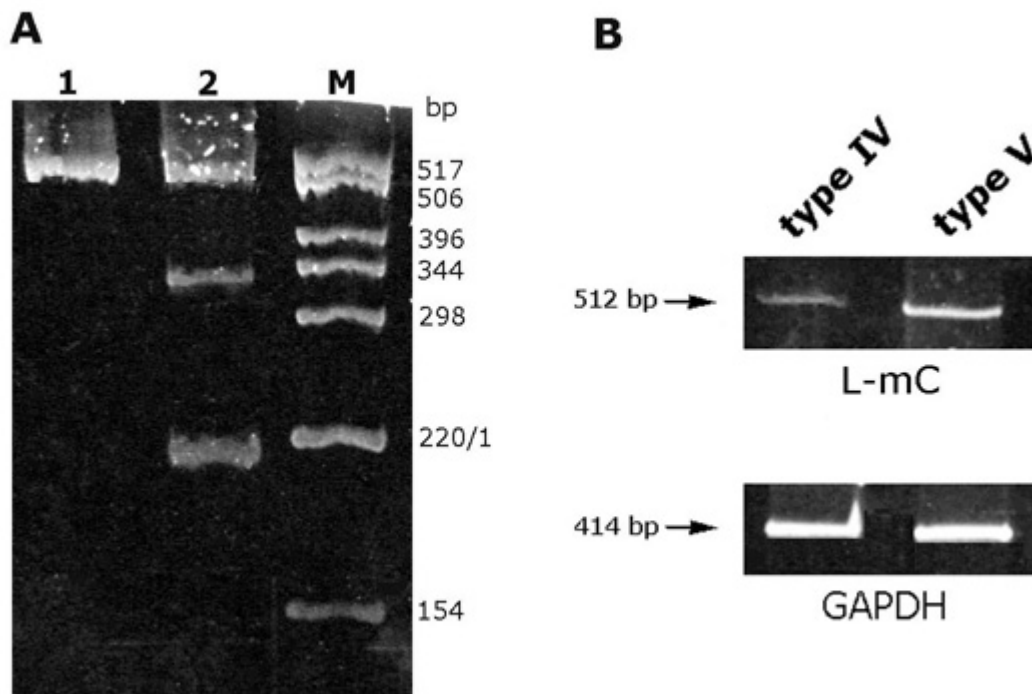
We then checked whether the observed increase of *L-mC* gene expression found was correlated to the presence of an enhanced amount of L-mC protein product. For this purpose, equal amounts of total protein extracts from cells cultured onto the two different collagen substrates were submitted to Western blot in the presence of an antibody specific for L-mC; the results obtained (fig. 6) showed a consistently stronger staining of L-mC in preparations of cells grown on type V collagen, than in those obtained from cells grown onto type IV collagen. Beta-actin was used as a control for gel loading.

Figure 6 Western blot analysis of L-mC content and control β-actin in lysates of 8701-BC cells grown onto the different collagen substrates.
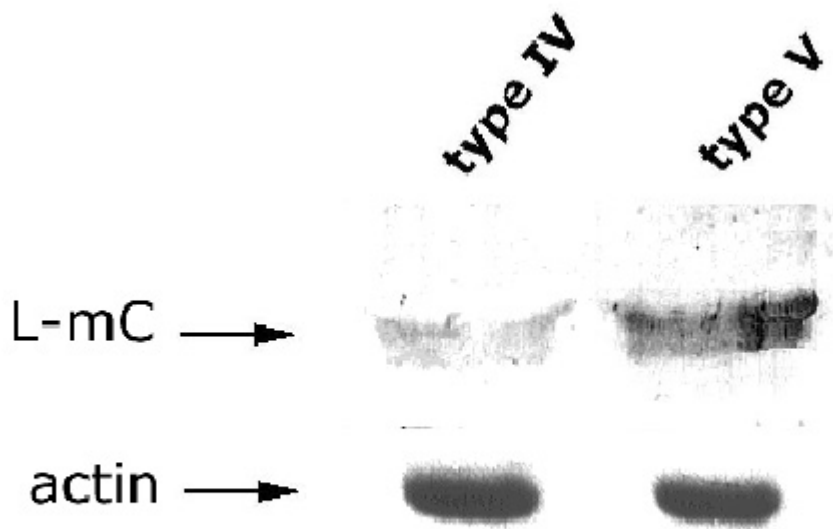
Inhibition of calpain and reduction of the extent of DNA fragmentation in 8701-BC cells grown onto type V collagen

To check whether calpain played a role in the apoptosis induced on 8701-BC cells by type V collagen, a cell-permeant inhibitor of calpain, calpain inhibitor I [42], was used at the concentration recommended by Squier *et al.* [34].

As shown in fig. 7, the addition of the calpain inhibitor to the medium of cells seeded on type V collagen-coated dishes resulted in an about 59% DNA fragmentation, both after 48 and 72 h from seeding; this represents approximately a 30% reduction of the amount of fragmented DNA with respect to untreated cells. Conversely, the calpain inhibitor exerted a "paradoxical" effect ($48 \pm 8\%$ of DNA fragmentation, i.e. about +30% *versus* untreated cells) only after 72 h when supplemented to the medium of cells seeded on type IV collagen-coated dishes. DMSO vector only was used in parallel control tests and it appeared not to modify significantly the extent of DNA fragmentation found in serum-free medium supplemented cultures.

Figure 7 Effect of calpain inhibitor I on DNA fragmentation in 8701-BC cells cultured onto either type IV or V collagen substrates for 48 and 72 h. Vertical bars indicate the s.e.m. of quadruplicate experiments. *$p < 0.05$ (treated versus control samples).
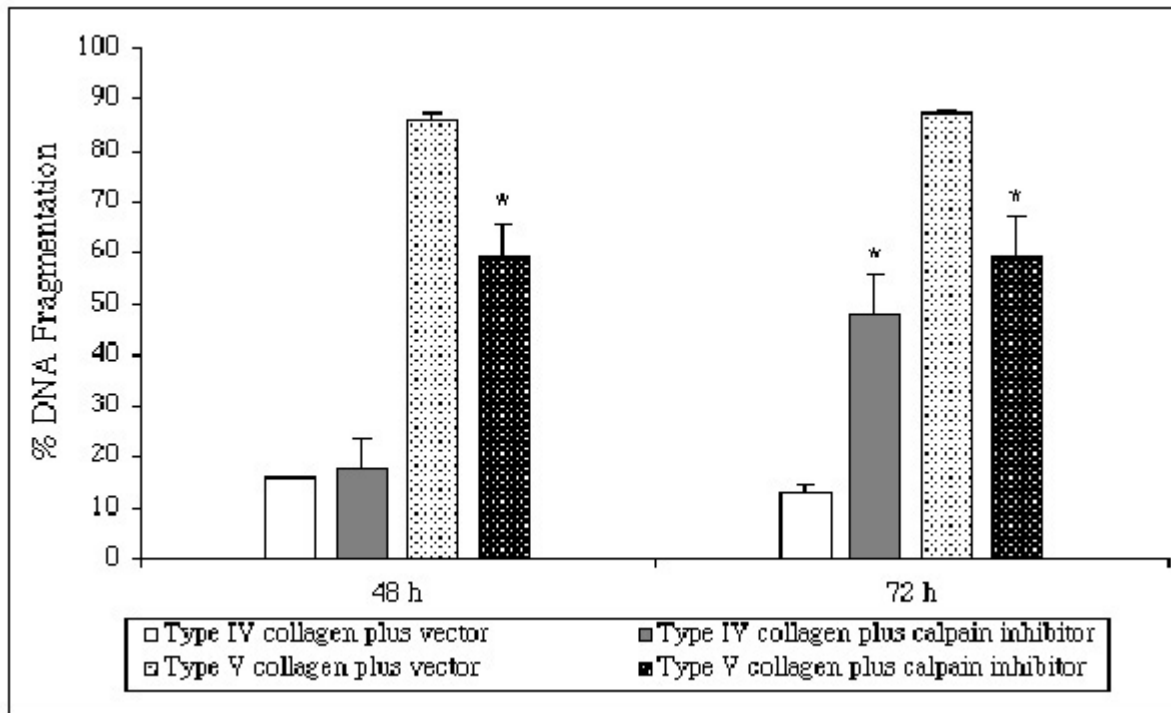
DISCUSSION

Type V collagen, a minor component of the multigenic collagen family, was originally detected in foetal membranes [43] and subsequently found, as a minor fraction of the total collagen, in several embryonic and adult tissues, where it occurs in a pericellular localization [44,45]. However, although the molecular and structural characteristics of type V collagen have been thoroughly investigated (see [46]), its biological role remains uncertain.

Some authors demonstrated that in mature tissues type V collagen molecules are buried within the major collagen fibrils [47,48] and therefore, in normal situations the epitopes for cell adhesion are not readily available to cells. Conversely, during tissue remodelling or in tumor invasion, enhanced deposition of collagen type V has been observed [19,49,50], and it is conceivable that it could become transiently available for cell adhesion, driving some specific cellular functions. However, the data so far available show that the cellular responses driven by this collagen appear inconsistent, suggesting differences among the model systems examined (e.g. tissues and cell types). For example, cell adhesion-promoting activity has been observed for normal smooth muscle cells [51,52], chinese hamster ovary cells [53], and some tumor cells [54]; conversely, anti-adhesive and anti-proliferative effects have been observed on endothelial cells [55,56,57], vascular smooth muscle cells [58] and some epithelial cells [59], besides the 8701-BC cancer cell line [2].

Present results confirm our previous observations on the "in vitro" inhibitory effect of type V collagen on cell proliferation, and offer an explanation of the biological mechanisms involved. Indeed, the

decrease of the expected growth rate in a given cell population may be due to two possible responses: a slow-down of the duplication rates or an increase rate of programmed cell death. The data presented here clearly support the second hypothesis. As it is known, apoptosis is a cell death program that produces specific morphological and biochemical modification, including loss of adherence to the substrate, cell shrinking in volume and chromatin condensation and separation into discrete masses, that produce a ladder-like pattern when the fragmented DNA is electrophoresed through agarose gels [60]. All the above-mentioned phenomenoma have been shown to occur in a large percentage of cells cultured onto type V collagen substrate.

Concerning the cell pathways involved in the apoptotic process, the knowledge is still fragmentary, but it is known that it invariably requires the activation of proteolytic cascades that are not yet well-defined. Cystein proteases, a family of non-lysosomal neutral proteases whose major members are calpain and caspases, have been considered as important intermediates in this cascade [61]. Some authors suggested that the proteolytic cascade is initiated after upstream caspase activation [62], others that calpains and the proteasome (a multicatalytic enzymatic complex) function synergistically downstream of caspases [63]. Concerning calpain substrates, cytoskeletal components (e.g. spectrin, fodrin, actin), membrane-linked proteins (e.g. Ca^{++} pump) and nuclear/cytosolic proteins [28], including cyclin D1 [64], have been recognised in a number of "in vitro" assays. Villa *et al*. [65] have suggested that the correlation between calpain-driven apoptosis and cytoskeleton might be ascribed to the efficacy of calpain to release DNase I from actin microfilaments, thus becoming more available for DNA fragmentation. Interestingly, as a support to this hypothesis in our model system, previous fluorescence microscopical analysis of actin in 8701-BC cells plated on type V collagen revealed the lack of a well-organized microfilament array [2], which was instead prominently present in cells cultured onto other collagen substrates [8,21].

Due to the importance of the apoptotic process in tissue homeostasis and in cancer biology and therapy, in the last few years a multiplicity of substances has been investigated as possible inducer/blockers of apoptosis (e.g. [66]), but, to our knowledge, this is the first documented indication that an endogenous tissue component, like type V collagen, is able to promote apoptosis in cancer cells. The expression of other components required for the apoptotic activation following 8701-BC adhesion onto type V collagen is currently under investigation

The present data substantiate our previous postulates that in cases of breast DIC 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 substrates [2,8,21,23,24]. It is therefore conceivable that the spatio-temporal derangement of stromal components may actively modulate neoplastic cell behavior and metastatic propensity, thus contributing to the selection and development of more or less malignant tumor phenotypes.

Acknowldgements

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