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Collagen-induced differential expression of an RNA polymerase subunit by breast cancer cells

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Abstract

It was previously reported that the stroma of ductal infiltrating carcinoma (DIC) of the human breast contains considerable amount of an embryo-foetal collagen type, OF/LB (onco-foetal/laminin-binding), and that adhesion of 8701-BC DIC cells onto OF/LB collagen substrates selectively promotes cell growth, motility, production of extracellular lytic enzymes and invasion "in vitro" if compared with other collagen species. To detect possible transcriptional differences for regulatory proteins following OF/LB collagen–cell interactions, we submitted RNA preparations from 8701-BC cells grown on collagen type I, IV and OF/LB to "differential display"-PCR in the presence of degenerate C_2H_2 zinc finger and protein tyrosine kinase domain oligonucleotide primers. We report that growth of 8701-BC cells on OF/LB collagen is consistently associated with the up-regulation of *hRPB17* gene, coding for an RNA polymerase subunit, as confirmed by conventional RT-PCR and Northern analyses.

Keywords: Collagen; Cell culture; Breast cancer; RNA polymerase

1. Introduction

It was previously reported that in cases of ductal infiltrating carcinoma (DIC), the desmoplastic stroma of the human breast is markedly modified in its ultrastructural appearance [1,2], and that qualitative changes in interstitial collagen composition can be found, the most remarkable being the reappearance of an embryo-foetal species of collagen, named OF/LB (onco-foetal/laminin-binding) for being undetectable in normal adult tissues [3] but present in the extracellular matrix (ECM) of both human umbilical cord and breast/colon carcinomas, and for its affinity to laminin in affinity chromatography [4]. 2D-electrophoretic analyses of OF/LB collagen demonstrated that this collagen species was composed of an unusually-acidic component, absent in other collagen types, and of two more basic components endowed with the same electrophoretic behavior of α_1(I) and α_1(III) collagen chains [4,5]. Evidence was also produced that OF/LB collagen, when used as a culture substrate for the DIC cell line 8701-BC [6], markedly promoted cell growth, motility, production of lytic enzymes of the ECM and invasion of reconstituted basement membrane, in opposition to what found for the other interstitial collagen types tested [7–11].

These collective data, demonstrating a prominent variation in 8701-BC cell phenotypic traits induced by OF/LB collagen, prompted a more detailed analysis of the effect of cell–collagen adhesion on gene expression. "Differential display" (DD)-PCR is a powerful technique which permits the isolation of cDNAs reverse-transcribed from mRNAs, allowing comparison of gene expression of differentially-treated samples; an ever-increasing list of variants of the original technique is available in the literature [12]. Previously, we identified the up-regulation of *HSP2A* and *MSF-B* in cells cultured onto OF/LB collagen substrates [13] utilizing the DD-PCR protocol published by Sokolov and Prockop [14]. In search for further differences in the expression patterns of mRNAs for other regulatory factors, in the present work we

Abbreviations: DD, differential-display; DIC, ductal infiltrating carcinoma; ECM, extracellular matrix; GAPDH, glyceraldehyde phosphate dehydrogenase; HSP2A, human protein phosphatase 2A regulatory subunit alpha isotype; MSF-B, human MLL septin-like fusion protein; OF/LB, onco-foetal/laminin-binding; PAGE, polyacrylamide gel electrophoresis; 2D, bidimensional.

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submitted RNA preparations from 8701-BC cells grown on different collagen substrates to DD-PCR in the presence of degenerate C_2H_2 zinc finger and protein tyrosine kinase domain oligonucleotide primers, as reported in [15]. The substrates tested were collagen type I, OF/LB and also type IV, the latter being the major component of the basement membrane which is the "natural" substrate of breast epithelial cells. Here we report that growth of 8701-BC cells onto OF/LB collagen substrate is associated with the up-regulation of *hRPB17* gene coding for one of the RNA polymerase subunits.

2. Materials and methods

2.1. Cells and substrates

8701-BC breast cancer cells were routinely cultured in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% foetal calf serum (Gibco) and antimycotic/antibiotics. Collagen type I and IV were purchased from Sigma (St. Louis, MO, USA), whilst purified OF/LB collagen, extracted from colon cancer tissue [4,5], was provided by Professor I. Pucci-Minafra (University of Palermo, Italy). Collagens were dissolved in 0.5 M acetic acid, sterilized with chloroform, plated at the concentration of 10 μg/cm^2 for 2 days and exhaustively neutralized with PBS just before cell seeding, as reported in [8]. 8701-BC cells were grown on collagen-coated dishes in serum-free medium for 48 h before RNA extraction.

2.2. RNA extraction, cDNA synthesis and DD-PCR

Total RNA was obtained from 8701-BC cells by extraction with TriPure™ Isolation Reagent (Roche, Mannheim, Germany). Two different RNA preparations from each experimental condition were pooled to make more pronounced any differences between the expression levels. Five micrograms of RNA were reverse transcribed with Superscript™ (Gibco, Gaithersburg, MD, USA) in the presence of 2 μg of random hexamers.

DD-PCR experiments were performed using the following primer pairs 5′-GG(ACTG)GAGAA(AG)CCCT (AT)(CT)GA(AG)TG-3′ (sense) and 5′-GA(CT)GT(CG) TGGTC CT(AT)TGG-3′ (antisense), which were designed on the consensus sequence established by aligning various C_2H_2 zinc finger DNA sequences, and on the protein tyrosine kinase domain, respectively [15]. PCR amplification was carried out in the presence of 2 U of recombinant *Taq* polymerase (Gibco) and 2 μl of the cDNA template. The first 15 cycles of each amplification followed a profile of 94 °C for 1 min., 37 °C for 30 s. and 63 °C for 3 min.; the subsequent 25 cycles were of the profile 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. PCR products were analyzed by 6% PAGE in a Protean II minigel apparatus (Bio-Rad) and visualized at 315 nm transillumination after staining with ethidium bromide.

2.3. Cloning and sequencing

The amplified cDNA fragment of interest was inserted in pCR™II vector using the TA cloning system (InVitrogen, San Diego, CA, USA), according to manufacturer's instructions. DNA sequencing of both strands of the cloned fragment was performed using Sequenase® v.2.0 (Amersham, UK) in the presence of ^{35}S-labeled dATP (NEN, Boston, MA, USA). Sequence ladder was visualized with Hyperfilm β-max films (Amersham). DNA sequence similarity was searched with the BLAST algorithm [16] available on-line at http://www.ncbi.nlm.nih.gov.

2.4. Conventional PCR

Conventional PCR was performed in the presence of 50 mM of two primers for hRPB17/hsRPB8 (accession numbers Z49199 and U37689), 5′-TTGACCGAGTGTC-TCGACTGC-3′ (sense) and 5′-CCCATAGGACACGTA-CGCAGA-3′ (antisense), designed with the Primer Selection software available on-line at http://alces.med.umn.edu, and specific for a 292 bp sequence from bases 143–435 of the transcript [17]; GAPDH was amplified in parallel. Preliminary assays were performed to determine the number of cycles to both keep amplification within the exponential phase and permit sufficient visualization of the PCR product. The cycle profile chosen was 94 °C for 2 min, followed by 20 cycles of 94 °C for 30 s, 49 °C for 45 s, 72 °C for 1 min (5 min during the last cycle). PCR products were analyzed by 6% PAGE and visualized at 254 nm transillumination after staining with SYBR Green I (Molecular Probes, Leiden, NL). The identity of the amplification product was checked by sequencing.

2.5. Generation of biotinylated probe and Northern blot

Twenty nanograms of purified PCR products were used as template for generation of biotinylated probe by unidirectional PCR [18] in the presence of 1 μM sense primer only and 0.035 mM biotin-16-dUTP (Roche). Fifteen micrograms of total RNA, extracted from 8701-BC cells cultured onto either type I, IV and OF/LB collagen, were electrophoresized in 1% agarose-formaldehyde gel as described in [19] and blotted onto positively-charged nylon membrane (Roche). Hybridization was performed according to a protocol available on-line (http://micro.nwfsc.noaa.gov/protocols/northernblot.html) in the presence of 100 ng of biotinylated probe. The chemiluminescent reaction was obtained by sequential addition of alkaline phosphatase-streptavidin and CSPD substrate, and recorded on Lumi-film (Roche). SigmaScan software (SPSS, USA) was utilized for the evaluation of pixel intensity of the chemiluminescent bands.

3. Results and discussion

RNA preparations obtained from 8701-BC cells grown onto different substrates were submitted to a DD-PCR analy-

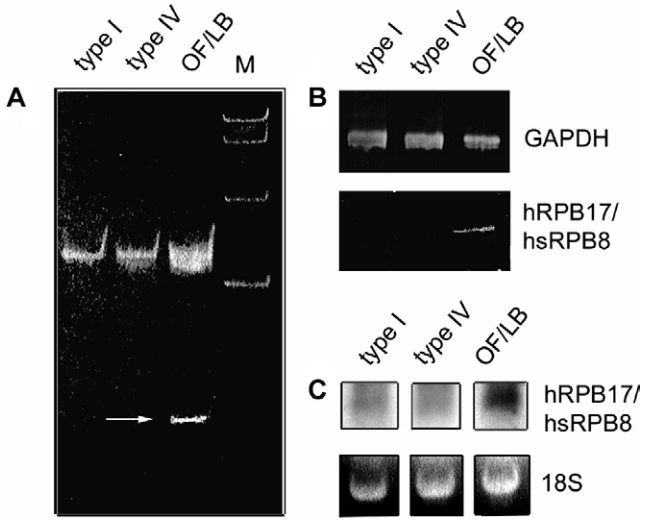

Fig. 1. A) DD-PCR of cDNA preparations from 8701-BC cells grown onto type I, type IV and OF/LB collagen substrates. A low-sized band of about 120 bp is selectively present in OF/LB sample (white arrow). M: size marker (λ DNA/*Hind*III, Gibco), 6% PAGE, ethidium bromide stain. B) PCR amplification of cDNA preparations from 8701-BC cells grown onto type I, type IV and OF/LB collagen in the presence of primers specific for GAPDH and hRPB17/hsRPB8 cDNAs, 6% PAGE, SYBR Green I stain. C) Upper: Northern blot hybridization of total RNA from 8701-BC cells grown onto type I, type IV and OF/LB collagen probed with biotinylated PCR product specific for hRPB17 transcript. Lower: ethidium bromide staining of the 18 S rRNA of the same samples as a control for gel loading, 1% agarose-formaldehyde electrophoresis.

sis in the presence of degenerate primers for zinc finger and protein tyrosine kinase domains. As shown in Fig. 1A, apart from some slowly-migrating bands common to the three different preparations although different in their intensity, cells cultured onto OF/LB collagen displayed a lower-sized distinctive band of about 120 bp, which was not detectable in the other electrophoretic lanes. The corresponding DNA fragment was cloned and sequenced, and homology was found in the databases available on-line between the sequence obtained and that of region 426–551 of the mRNA for the 17-kDa human RNA polymerase subunit known as hRPB17/hsRPB8 (accession numbers Z49199/U37689).

Conventional PCR and Northern analyses were performed to confirm the data obtained. For PCR assays, RNA samples from 8701-BC cells were subjected to PCR analysis in the presence of primers specific for hRPB17/hsRPB8 mRNA, amplifying GAPDH in parallel as a control for starting material. As shown in Fig. 1B, at low cycle number the amplification product for hRPB17/hsRPB8 was found to be present only in the cDNA preparation from cells grown onto OF/LB collagen substrate; nonetheless, at higher number of PCR cycles an amplification signal was present also in the other cDNA preparations (not shown). Northern analysis was performed using a biotinylated probe specific for hRPB17 mRNA and supported the data obtained with PCR; as shown in Fig. 1C, the hybridization signal was present in all the lanes but its intensity was higher in the presence of RNA from cells cultured onto OF/LB collagen. Normalization of hRPB17/hsRPB8 band intensities for those of 18S RNA indicated that OF/LB collagen was able to promote the up-regulation of the RNA polymerase subunit by about 2- and 2.37-fold with respect to type I and type IV collagen.

It is known that eukaryotic RNA polymerases are heteromultimeric enzymatic complexes composed of at least 12 subunits ranging from less than 10 to 220 kDa [20,21], some of them endowed with extensive aminoacid sequence conservation even comprising archaebacterial homologs, whose functions played are still to be fully elucidated. The subunit Hs8, encoded by *hRPB17/hsRPB8* gene is a 17-kDa 191-aminoacid polypeptide showing a rather acidic p*I* (4.34), shared by the three human RNA polymerases. Very limited data exist on the biological characterization of this protein; in particular, experiments of heterocomplementation "in vivo" of some human polymerase subunit genes in *S. cerevisiae* have shown that in the absence of its yeast homolog, *hsRPB8* gene can support normal cell growth, albeit with a thermosensitive phenotype [17,22]. This supports the hypothesis of a fundamental and wide-spread role conceivably played by *hRPB17/hsRPB8* product in the efficiency and regulation of cell cycle.

It is generally acknowledged that the ECM plays a critical role during growth, differentiation and carcinogenesis through a network of "signal" molecules which profoundly influence cell behavior, and ECM-mediated control of gene expression has been observed in some "in vitro" models of animal and human mammary tissue, e.g. the laminin-promoted up-regulation of *β-casein* and the modulation of *estrogen receptor-α* expression and function by seeding onto collagen-IV and laminin-1 substrates [23,24]. However, to our knowledge the present results suggest for the first time that adhesion of a breast cancer cell line to substrates of a peculiar collagen species of breast cancer ECM, i.e. OF/LB, may exert specific effects on the transcription machinery by identifying an essential component of the general transcription apparatus, hRPB17, whose expression is up-regulated with respect to cells grown onto type I and IV collagen substrates. Although the molecular mechanism underlying the observed phenomenon and its biological implications remain to be determined, we cannot exclude that this up-regulation is related, at least in part, with the drastic changes in 8701-BC cell phenotypic expression when cultured onto OF/LB collagen substrate, i.e. the advantage in cell proliferation, locomotion, ECM enzyme release and invasive potential [7–11].

In conclusion, *hRPB17* represent a novel example of gene differentially-expressed by 8701-BC cells in response to OF/LB collagen, thereby increasing the list of genes whose expression is controlled by adhesion onto this collagen substrate [13]; moreover, up-regulation of *hRPB17* gene expression might represent an additional molecular marker for the identification of overtly-malignant cells in breast cancer. The validation of this latter hypothesis will require further study.

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