Extracellular matrix regulation of PTHrP and PTH/PTHrP receptor in a human breast cancer cell line

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Abstract It was previously reported that 8701-BC breast cancer cells express the gene for parathyroid hormone-related peptide (PTHrP) and its cognate receptor (PTHrP-R), and release immunoreactive PTHrP in the extracellular medium; it was also found that PTHrP, in turn, exerts a role on the proliferative and invasive behavior in vitro of the same cell line. On the other hand, evidence has been produced that adhesion of 8701-BC cells onto different collagen substrates influences in various ways a number of phenotypic expressions, such as cell growth, motility, invasion of reconstituted basement membrane and production of lytic enzymes of the extracellular matrix (ECM). In light of these previous data, we have examined whether substrates of either reconstituted basement membrane or representative collagen components of the breast tumor stroma (type I, V and OF/LB) might (i) regulate the *PTHrP* promoter usage and mRNA splicing patterns, (ii) modulate quantitatively the extracellular release of immunoreactive PTHrP (iPTHrP), and (iii) affect the expression of *PTHrP-R*. The results obtained give evidence that (i) 8701-BC cells are able to utilize different start sites and mRNA splicing patterns for *PTHrP* transcription; (ii) 'structural' components of the stroma, such as collagens, are by themselves capable of controlling both the expression pattern of the *PTHrP* gene and the extent of extracellular release of iPTHrP, and (iii) *PTHrP-R* expression can be up- or down-regulated in response to the ECM substrate present. These data demonstrate that *PTHrP* and *PTHrP-R* expression by 8701-BC neoplastic cells can be modulated by ECM molecules, indirectly supporting the active participation of stromal collagen composition in the regulation of PTHrP-controlled circuits which may play a role in carcinogenesis.

Key words: Extracellular matrix; Breast cancer; Gene expression; Parathyroid hormone-related peptide; Parathyroid hormone-related peptide receptor

1. Introduction

Previous ultrastructural and biochemical investigations have shown that in cases of ductal infiltrating carcinoma (DIC), a highly invasive tumor histotype of the human mammary gland, the desmoplastic stroma, apart from displaying marked modifications in its architecture, undergoes conspicuous quantitative and qualitative changes in its collagen composition. These changes chiefly consist of the enhanced deposition of collagen type V and the re-appearance of OF/LB (onco-fetal/laminin-binding) collagen. The latter is an embryo-fetal collagen species absent in normal adult tissues but detectable also in human umbilical cord and colon carcinoma tissues, which is composed of three α_1(I)-sized chains one of which displaying an unusually acidic p*I* [1–4]. In addition, when cell–collagen interplays were studied using the 8701-BC breast cancer cell line [5] as an in vitro model of neoplastic cell population from primary DIC, evidence was produced that adhesion of cells onto different collagen substrates influenced in varying ways a number of phenotypic expressions related to neoplastic progression in vivo, such as cell growth, motility, invasion of reconstituted basement membrane and production of lytic enzymes of the extracellular matrix (ECM) [6–10].

We have recently shown that also the production of parathyroid hormone-related peptide (PTHrP) by breast cancer cells can be conceivably considered one of the key elements instrumental in promoting carcinogenesis [11,12]. PTHrP is the product of a gene exhibiting a complex organization in humans, with three transcriptional start sites, i.e. two TATA boxes (P1 and P3) and a GC-rich region (P2), and nine exons undergoing alternative splicing and producing mRNA variants with likely differential spatio-temporal expressions. The deduced encoded polypeptides, of either 139, 141 or 173 amino acids, display sequence homology with PTH at the extreme N-terminus which allows the binding to the same G protein-linked receptor (PTHrP-R). In recent years, evidence was brought that PTHrP may be a polyhormone undergoing proteolytic processing, possibly tissue-specific, into several smaller bioactive forms, some of which have been either immunodetected or isolated from conditioned media of normal and neoplastic cells. The polyproteic and almost ubiquitous nature of PTHrP has therefore led to the concept that, apart from its classically recognized PTH-like role played in establishing the humoral hypercalcemia of malignancy, discrete fragments of this protein may function as local autocrine/paracrine modulators mediating growth factor-like roles through the PTHrP-R and other still unknown receptor(s) (see [13] for a review). Interestingly, the *PTHrP* gene has been described as a downstream target for *ras* and *src* and an upstream element of the *Bcl-2* and c-*fos* signalling pathways, thus supporting its involvement in the control of cell proliferation, differentiation and survival [14–16]. In addition, *PTHrP-R* expression by bone cells has been found to be down-regulated in the presence of ECM, suggesting a role of the latter in the modulation of PTHrP/PTHrP-R interactions [17].

It was previously reported that 8701-BC cells express the

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genes for *PTHrP* and its cognate receptor and release immunoreactive PTHrP (iPTHrP) fragments in the extracellular medium [11,12,18]. In addition, PTHrP 1–34, 67–86 and, to a minor extent, 107–139 have been described as anti-mitogenic but invasion-promoting for the same cell line [11]. The present study was designed to further expand our knowledge on tumor cell–ECM interactions in vitro, by examining whether substrates made of either reconstituted basement membrane components or selected collagen species present in DIC stroma (i.e. type I, V or OF/LB) which markedly alter cell behavior, might (i) modulate quantitatively the extracellular release of iPTHrP, (ii) regulate the *PTHrP* promoter usage and mRNA splicing patterns, and (iii) affect the expression of *PTHrP-R*.

2. Materials and methods

2.1. Cell cultures

Collagen types I and V were purchased from Sigma (St. Louis, MO, USA). OF/LB collagen was extracted from colon cancer tissue as previously described [3,4] and the purity of the preparation was checked by 2D-polyacrylamide gel electrophoresis (2D-PAGE) before utilization (Fig. 1). Collagens were dissolved in 0.5 M acetic acid, sterilized with chloroform as reported in [7], plated in 25 cm^2 flasks at a concentration of 10 μg/cm^2 for 2 days and exhaustively neutralized with phosphate buffered saline just before cell culturing. Matrigel, purchased from Collaborative Res. (Bedford, MA, USA), was diluted to a concentration of 250 μg/ml and 1 ml was plated in 25 cm^2 flasks and allowed to gel overnight.

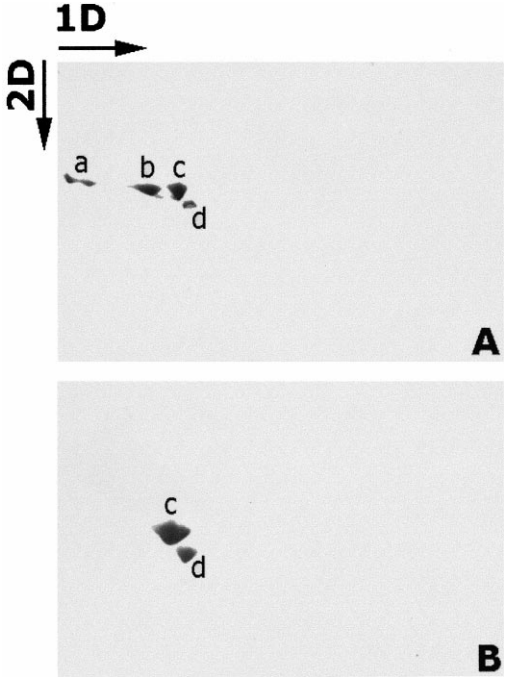


Fig. 1. 2D-PAGE analysis of OF/LB collagen used in the present study. Collagen was extracted from a biopsy fragment of the tumor core and of the adjacent colonic mucosa by mildly acidic pepsin digestion and OF/LB collagen fractionated by several cycles of differential precipitation at pH 7.2 [3,4]. The preparation obtained from the neoplastic tissue (A), subsequently used for the biological assays described, shows the presence of the characteristic acidic chain of OF/LB collagen appearing as a twin spot (a), and of the two other components of this collagen species migrating as α_1(III)- and α_1(I)-like chains (b and c). The component present in band d displays the migratory behavior of the α_2(I) collagen chain, although recent biochemical evidence has demonstrated the occurrence of sequence overmodifications in tumoral α_2(I) collagen chains with respect to the 'regular' counterpart [38]. As expected, no component of OF/LB collagen can be observed in the preparation from the adjacent 'normal' tissue (B), used as a control for collagen extraction. First dimension (1D): non-equilibrium pH gradient gel electrophoresis (NEPHGE); second dimension (2D): SDS-PAGE. Coomassie stain.

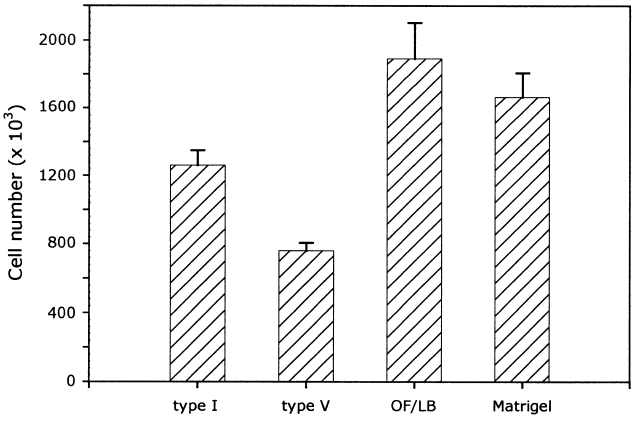


Fig. 2. Effect of the different ECM substrates on the proliferative behavior of 8701-BC cells 48 h after plating. The vertical bars indicate the S.E.M.

8701-BC cells [5] were routinely cultured in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% fetal calf serum (Gibco) and antibiotics. Cells were detached with 0.1% EDTA, plated in substrate-coated flasks at a concentration of 10^6/flask in serum-free conditions and allowed to grow for 2 days. At the end of the assay, the conditioned medium (CM) was collected, centrifuged at low speed to discard cell debris, lyophilized and stored at −20° until used. Cell number was determined by counting with a hemocytometer.

2.2. Midregional iPTHrP radioimmunoassay (RIA)

For this assay, 100 μl goat antiserum (1:8000) raised against amino acids 53–84 and 100 μl reconstituted CM were incubated under non-equilibrium conditions. Synthetic human PTHrP 1–86 (Bissendorf Biochem., Hannover, Germany) was used as standard and for tracer preparation [19]. The assay had a detection limit of 5 pmol/l. The results of RIAs were normalized to actual cell number and expressed as pmol iPTHrP/10^6 cells.

2.3. RNA extraction, cDNA synthesis and polymerase chain reaction (PCR)

Total RNA from 8701-BC cells cultured on the different substrates was isolated with TRI reagent (Roche, Mannheim, Germany). Aliquots of 5 μg of RNA from at least two different preparations were reverse-transcribed using the SuperScript kit (Gibco, Gaithersburg, MD, USA) in the presence of random hexamers, according to the manufacturer's instructions. The semi-quantitative PCR amplification was carried out as described by Brandt et al. [20] in an UNO Thermoblock (Biometra, Göttingen, Germany) using 100 ng of RNA/cDNA diluted with 0.5 mM dNTPs, 0.5 μM of each primer, 1 unit of r*Taq* DNA polymerase (Pharmacia, Uppsala, Sweden) and 0.5 μCi [^{32}P]dCTP (ICN, Thame, UK). The thermal cycle used was a denaturation step of 96°C for 3 min and a 5 min 'ramp' time to the annealing temperature for 2 min, followed by 30 cycles of 95°C for 15 s, the annealing temperature for 30 s and 72°C for 45 s. GAPDH was amplified in parallel and used as inter-sample control; radiolabelled amplification products of actin, GAPDH, transforming growth factor β1 (TGFβ1) and urokinase-plasminogen activator (uPa) were employed as size markers. The primers used and the predicted size of their amplification products are listed in Table 1 along with related references. PCR products were analyzed by 8% non-denaturing polyacrylamide gel electrophoresis in a Mini-Protean II apparatus (Bio-Rad, Richmond, CA, USA) and visualized after exposure of the dried gels to Kodak BioMax films with intensifying screen at −80°C. SigmaGel 1.0 software (Jandel Scientific, USA) was utilized for the size evaluation of the electrophoretic bands.

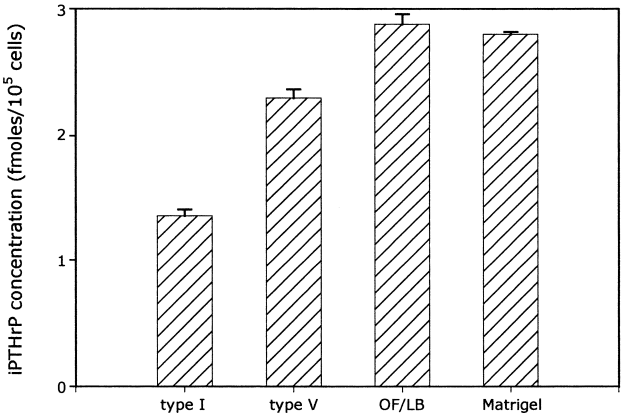


Fig. 3. Effect of the different ECM substrates on iPTHrP production by 8701-BC cells 48 h after plating. The vertical bars indicate the S.E.M.

2.4. Statistics

Data are presented as mean ± S.E.M. of either three (for proliferation) or six (for iPTHrP dosage) different experiments; a software-assisted Student's *t*-test was performed (SigmaStat v.2, Jandel, USA) and $P < 0.05$ was taken as the minimal level of statistical significance.

3. Results

When Matrigel, type I, V or OF/LB collagen are used as substrate cultures, 8701-BC cells undergo modifications of their proliferative behavior. In particular, as shown in Fig. 2, OF/LB collagen substrate and Matrigel are equally strong promoters of 8701-BC cell growth (average of about +89% and +67% with respect to the initial cell concentration; $P > 0.05$ between the two sets of data) in opposition to type I (−33.3 ± 3% versus OF/LB collagen; $P = 0.019$) and, mainly, to type V collagen substrates (−59.8 ± 1.5% versus OF/LB collagen; $P < 0.001$).

Therefore, we examined whether the different substrates were also able to modulate the production of iPTHrP, subjecting cell CMs to a RIA specific for a mid-regional domain of PTHrP which was proven to give reliable results with the 8701-BC cell line [11]. The data reported in Fig. 3 indicate that the release of PTHrP can be strongly influenced by adhesion onto different substrates. In particular, after normalization of the data to the actual cell numbers, variable in the different culture conditions as already reported, OF/LB collagen and Matrigel are found to be the most efficient inducers of PTHrP secretion ($P > 0.05$ between the two sets of data).

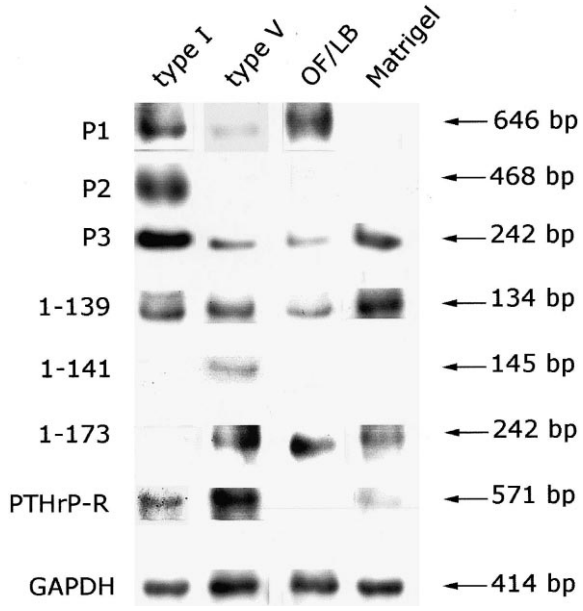
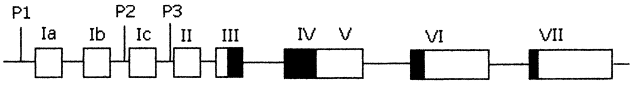


Fig. 4. Panel of semi-quantitative PCR analyses of PTHrP promoter usage and splicing pattern, and of PTHrP-R expression, representative of triplicate assays. The amplification was carried out in the presence of [^{32}P]dCTP and the products were separated by 6% PAGE in parallel with labelled controls (not shown) and autoradiographed at different exposure times in order to optimize the visibility of all the bands. GAPDH was used as inter-sample loading control.

Table 1
Sequence of primers used for PCR amplification

Transcript detected	Oligonucleotides	Product size (bp)	Reference
PTHrP P1	5′-AGGTACCTGCTTTCTAATA-3′ 5′-TGCGATCAGATGGTGAAGGA-3′	739, 646, 406	[21]
PTHrP P2	5′-TTCTCCGGCAGGTTTG-3′ 5′-TGCGATCAGATGGTGAAGGA-3′	468	[21]
PTHrP P3	5′-GTTGGAGTAGCCGGTTGCTA-3′ 5′-TGCGATCAGATGGTGAAGGA-3′	242	[21]
PTHrP 1–139	5′-AAACGGCGAACTCGCTCT-3′ 5′-AGGCTACGGGCCAGAGAAG-3′	134	[22]
PTHrP 1–141	5′-AAACGGCGAACTCGCTCT-3′ 5′-TGTCCTTGGAAGGTCTCTG-3′	145	[22]
PTHrP 1–173	5′-AAACGGCGAACTCGCTCT-3′ 5′-CCTATCTGTAGCAGAGTCAA-3′	242	[22]
PTHrP-R	5′-AGGAACAGATCTTCCTGCTGCA-3′ 5′-TGCATGTGGATGTAGTTGCGCGT-3′	571	[12]
GAPDH	5′-CATGGAGAAGGCTGGGGCTC-3′ 5′-CACTGACACGTTGGCAGTGG-3′	414	[21]
β-Actin	5′-GTGGGGCGCCCCAGGCACCA-3′ 5′-CTCCTTAATGTCACGCACGATTTC-3′	548	[18]
TGFβ1	5′-GCCCTGGACACCAACTATTGC-3′ 5′-GCTGCACTTGCAGGAGCGCAC-3′	336	[18]
uPa	5′-ATGAGAGCCCTGGCGCGCCTG-3′ 5′-TTTTTGAATCTATTTCACAGTGCTGCCCTCCGAATTTT-3′	198	[18]

Table 2
(Top) Structure of the human *PTHrP* gene (filled boxes = coding sequences, open boxes = 5′- and 3′-untranslated regions [23]) and (bottom) synopsis of potential mRNA species synthesized by 8701-BC cells onto the different substrates, as evinced by PCR data



Type I collagen	Ia-Ic-III-IV-V	Type V collagen	Ia-Ic-III-IV-V
	Ic-II-IC-V		Ia-Ic-III-IV-VI
	II-III-IV-V		Ia-Ic-III-IV-VII
			II-III-IV-V
			II-III-IV-VI
			II-III-IV-VII
OF/LB collagen	Ia-Ic-III-IV-V	Matrigel	II-III-IV-V
	Ia-Ic-III-IV-VI		II-III-IV-VI
	II-III-IV-V		
	II-III-IV-VI		

Conversely, the iPTHrP amount decreases in the CM of cells cultured on type V ($-19.8 \pm 2.7\%$ versus OF/LB collagen; $P = 0.005$) and, more dramatically, type I collagen substrate, the latter being responsible for a massive drop of iPTHrP in the extracellular milieu ($-53 \pm 2\%$ versus OF/LB collagen; $P < 0.005$).

Subsequently, we analyzed PTHrP promoter usage and mRNA splicing patterns in the presence of the various culture substrates. As shown in Fig. 4, 8701-BC cells are able to utilize each of the three different transcriptional start sites and the three 3′ splicing pathways with different arrays in the presence of the substrates examined. Noteworthy, the unique size of the amplification product in the presence of P1-specific primers is indicative of the generation of exon I-III-V-VI isoform only, and the use of the P3 initiation site appears to be widespread whilst that of P1 and P2 is more restricted, in accordance with [21]. In particular, in the presence of type I collagen all the promoters were used with a P3 > P2 > P1 pattern; no other substrate elicited the usage of the GC-rich promoter since in the presence of type V and OF/LB collagen only P1- and P3-specific products were evident (P3 > P1 for type V collagen and P1 > P3 for OF/LB collagen). When cells were seeded onto Matrigel, only the downstream TATA box was transcriptionally active. Concerning the PTHrP species, the 1–139 variant was present in all the cDNA preparations and was the only species synthesized by cells onto type I collagen substrate. Moreover, the 1–173 isoform-specific PCR product was found in all but type I collagen-derived cDNA preparation and the 1–141 isoform-specific PCR product only in type V collagen-derived samples and in smaller amount than the other two species. Table 2 reports the potential mRNA species arising from alternative splicing in response to 8701-BC cell culturing on the different substrates in light of both the present results and the organization of the human *PTHrP* gene [23].

Further, the results concerning the expression of *PTHrP-R* indicated its up-regulation in cells plated onto type V collagen mainly and, to a lesser degree, type I collagen compared to cells grown on Matrigel and OF/LB collagen, in which no amplification signal was detectable, at least under the experimental conditions used.

4. Discussion

It is widely recognized that ECM, due to its complex and varying nature, may bear multiple instructive signals and elicit a wide range of dissimilar cell responses, which are of primary relevance during development, differentiation and cancer and whose molecular mechanisms are still mostly unknown. *PTHrP* expression is one of those biological activities which has been extensively studied in in vivo and in vitro systems because of its massive involvement in several normal and neoplastic developmental processes (e.g. [23–27]). Moreover, Behrendtsen et al. [28] have shown that PTHrP produced during some differentiation stages of the mouse embryo is able to modify integrin expression and function and the related cell response to extracellular ligands, thus actively cooperating to drive cell–ECM interplays.

In the mammary gland, PTHrP is expressed in both the resting and the lactation phase albeit to different extents [29] and appears to influence the state of organ proliferation and differentiation; in fact, PTHrP oversecretion obtained by transgene technology has been proven to inhibit ductal elongation and branching [30]. Ferrari et al. [31] and Sebag et al. [32] have demonstrated that certain growth factors and hormones, e.g. TGFβ, IGF-I, EGF, prolactin, are capable of modifying the rate of PTHrP production by normal human mammary epithelial cells in vitro.

We have already demonstrated the in vitro anti-proliferative and pro-invasive effect exerted on the carcinoma cell line 8701-BC and some derived clones by PTHrP [11,12], which has been therefore regarded also as one of the key elements potentially involved in mammary tumorigenesis in vivo. Nonetheless, the regulation of *PTHrP* and *PTHrP-R* expression and iPTHrP secretion by breast cancer cells has been poorly investigated, the unique data being those regarding the effect of phorbol ester, steroid hormones and anti-estrogens on KPL-3C and MCF7 cell lines [33–35].

To the best of our knowledge, here we report the first evidence that: (i) 8701-BC cells are potentially able to utilize different start sites and mRNA splicing patterns for *PTHrP* transcription; (ii) extracellular macromolecules of the stroma, even in the absence of biologically active soluble factors added to cell medium, are by themselves capable of controlling both the expression pattern of *PTHrP* gene and the extent of extracellular release of PTHrP by the same cell line; and (iii) *PTHrP-R* expression can be up- or down-regulated by the ECM substrate present. The last two points further support our hypothesis of the existence of diverse binding activities for ECM molecules on 8701-BC cell surface ([36], and work in progress). Interestingly, only one out of the four substrates tested induced P2 expression: this finding has a twofold significance since it strengthens the principle of ECM-driven cell modulation, and also confirms previous data by Southby et al. [21] on the scarcer utilization of the P2 (and P1) than the P3 site, which we could explain on the basis of the requirement of select ligands bound to cell surface.

An intriguing biological observation arising from the present study is the apparent correlation between the powerful down-regulation of *PTHrP-R* expression and the active promotion of cell proliferation operated by OF/LB collagen and Matrigel substrates, in opposition to type I and, mainly, type V collagen substrates. We have previously reported that the PTHrP 1–34 fragment is able to restrain the growth of the

8701-BC cell line and of the derived clonal sublines expressing PTHrP-R [11,12]. It can therefore be hypothesized that a growth-restraining behavior, such as that imparted by type V collagen to 8701-BC cells, could be more seemingly related to the ability to bind N-terminal PTHrP and install an autocrine effect than to the actual amount of iPTHrP released, which cannot be correlated with cell proliferation. Interestingly, recent data (Pucci-Minafra et al., submitted) have revealed the apoptosis-promoting effect of collagen type V on a conspicuous fraction of 8701-BC cells and, therefore, it will be worthwhile to examine if the observed modifications on *PTHrP* and *PTHrP-R* expression and production may be related to the gene activation cascade of the death program. On the other hand, whether the substrate-dependent modulation of the expression of the different species of transcripts or protein isoform may have some biological significance in our model system remains to be elucidated.

In conclusion, we have produced evidence that not only the proliferative behavior but also the expression of the *PTHrP*/*PTHrP-R* pair by a DIC cell line is modulated by stromal components and that the two phenotypic aspect are possibly associated. Although caution must be exercised in extrapolation of in vitro results to the in vivo situation, the data obtained further support the postulate that local ECM composition may be a crucial regulatory parameter for malignant ingrowth. The present findings are in fact consistent with the hypothesis that both permissive and inhibitory pathways may be present in the basal lamina and stromal compartments of the affected mammary tissue governing regulation of PTHrP and PTHrP-R expression and production to different degrees. These pathways, subjected to a dynamic and zonal turn-over of synthetic and degradative events (e.g. [37]), conceivably participate in feeding or restraining the autocrine/paracrine circuits which are likely to exist between PTHrP and sensitive breast cancer (and other) cells; the resulting differential effects on cell behavior might contribute in concert with other microenvironmental signals to control tumor growth and differentiation.

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