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Heparanase Expression in Circulating Lymphocytes of Breast Cancer Patients Depends on the Presence of the Primary Tumor and/or Systemic Metastasis[1]

Thérèse Rachell Theodoro, Leandro Luongo de Matos*, Aleksandra Vanessa Lambiasi Sant Anna†, Fernando Luiz Affonso Fonseca†, Patrícia Semedo‡, Lourdes Conceição Martins§, Helena Bonciani Nader‡, Auro Del Giglio† and Maria Aparecida da Silva Pinhal**

*Departamento de Bioquímica, Faculdade de Medicina do ABC, São Paulo, Brazil; †Departmento de Oncologia, Faculdade de Medicina do ABC, São Paulo, Brazil; ‡Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, Brazil; §Departamento de Saúde Comunitária, Faculdade de Medicina do ABC, São Paulo, Brazil

Abstract

Heparanase is an endo-β-glucuronidase that is capable of degrading heparan sulfate chains of proteoglycans, generating a variety of bioactive molecules such as growth factors and chemotactic and angiogenic agents. The expression of heparanase was investigated in the peripheral blood mononuclear cell fraction (PBMC) of 30 patients with breast cancer and 20 healthy control women by reverse transcription–polymerase chain reaction (RT-PCR) and immunocytochemistry. PBMC samples from all breast cancer patients at study entry showed the expression of heparanase, whereas no expression was observed for healthy women. Immunocytochemistry analysis demonstrated that heparanase was expressed in lymphocytes of breast cancer PBMC. Throughout follow-up, heparanase expression by RT-PCR decreased significantly after surgery in patients treated with neoadjuvant chemotherapy (*P* = .002) and after tamoxifen treatment (*P* = .040), whereas it increased significantly with the advent of systemic metastasis (*P* = .027). *In vitro*, either serum from breast cancer patients or a medium originated from coculture experiments of MCF-7 cells and lymphocytes from healthy women stimulated heparanase expression in normal lymphocytes. The results suggest that there is a tumor-inducing effect on heparanase expression by lymphocytes present in the PBMCs of breast cancer patients, which depends, in turn, on the interaction between a tumor and normal lymphocytes.

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Introduction

Solid tumors have three compartments: malignant cells, microcirculation, and stroma. There is a close interchange among all these components through paracrine and autocrine mechanisms involving cells from all these three compartments. As a result of this intricate cellular and humoral network, there is a change in the expression of various genes of cells belonging to all these three compartments, which act in concert and are responsible for malignant cell proliferation, apoptosis, angiogenesis, tumor cell invasion, and metastasis [1]. In addition to normal cells present in the stroma, breast cancer and other solid tumors also have variable amounts of tumor-infiltrating lymphocytes (TILs). In fact, breast cancer tumors infiltrated more prominently by TILs seem to have better prognosis [2]. Therefore, it is possible that TILs, in addition to their immune-related functions [3], may actively participate in the aforementioned network by actively secreting and responding to cytokines produced by tumors, endothelial cells, or stromal cells [4,5].

Heparan sulfate proteoglycans are found in extracellular matrices and on cell surfaces, playing critical functions in cell–cell and cell–matrix interactions [6,7]. In fact, transmembrane heparan sulfate proteoglycans (syndecans) are emerging as molecules that mediate cell interactions with components of the microenvironment that control cell shape, adhesion, proliferation, and differentiation [8,9]. Additionally, cell-associated heparan sulfate can potentiate the interaction of soluble growth factors with cell surface receptors, and its binding can also protect growth factor cleavage by proteolytic enzymes [10,11]. Furthermore, heparan sulfate proteoglycans are also prominent components of endothelial cells [12] and the basement membrane [13].

Address all correspondence to: Maria Aparecida da Silva Pinhal, Rua Dona Brígida, 31, 112, São Paulo 04111-080, Brazil. E-mail: maspinhal@yahoo.com.br

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Heparanase is an endo-β-glucuronidase that is capable of degrading heparan sulfate chains of proteoglycans, a key component of the extracellular matrix and the basement membrane. The oligosaccharides so generated lead to the release of a variety of bioactive molecules, such as growth factors, chemotactic agents, and angiogenic agents, which are then deposited in the extracellular matrix and basement membrane. These molecules can stimulate cell proliferation, increase cell survival, and promote angiogenesis, morphogenesis, and vascularization [14]. Fragments of heparan sulfate generated by heparanase can also induce the maturation of dendritic cells and activate macrophages, thereby stimulating the release of factors such as IL-1, IL-6, and prostaglandin E_2, which modulate immune cell responses [15,16]. Furthermore, protein or messenger RNA (mRNA) expression of heparanase has been identified in various cancer cells, and its overexpression in tumor cells has also been reported to correlate with metastatic potential and poorer prognosis [17,18].

In the present study, the expression of heparanase in the peripheral blood mononuclear cell (PBMC) fraction of breast cancer patients was analyzed serially with semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). We also report a series of *in vitro* assays to investigate possible mechanisms of the alterations in heparanase expression found in these patients. A panel of other proteins that are commonly altered in tumors—such as those involved in DNA mismatch repair (hMLH1, hMLH2, hPMS1, and hPMS2) [19] and those involved in cell proliferation and apoptosis (PCNA and P53) [20,21]—was also investigated.

Patients and Methods

Patients

Blood samples from 30 patients with histologically confirmed breast cancer were obtained after informed consent had been granted. From these patients, serial samples were collected at 3-month intervals before, during, and after systemic treatment (13 adjuvant, 12 neoadjuvant, and 5 palliative). Three patients initially received hormones (two adjuvantly and one palliatively). The chemotherapy combinations used in these patients were as follows: 5-fluorouracil, adriamycin, and cyclophosphamide; adriamycin and cyclophosphamide; and cyclophosphamide, methotrexate, and 5-fluorouracil. Peripheral blood samples were also collected at 3-month intervals from 20 healthy control women without any relevant previous medical history. The present study conformed to the regulations of The Human Ethic Research Committee at our institution, ABC School of Medicine (project no. 2000/04681-2).

mRNA Extraction and RT-PCR Analysis

Peripheral blood samples (20 ml) were collected every 3 months using EDTA. Mononuclear fractions were prepared using Ficoll Hypaque gradient (Teknica, Durham, NC). A total of 1×10^6 lymphocytes/ml was submitted to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The integrity of total RNA was checked electrophoretically and quantified spectrophotometrically. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA in a 25-μl reaction (Invitrogen) containing 1 μl of oligod(T)18 (0.5 μg/ml), 1 μl of dNTPs (10 mM), and 1 of μl reverse transcriptase (Invitrogen) Moloney Murine Leukemia Virus (M-MLV), and the reaction was performed at 42°C for 30 minutes. For PCR amplification, 2 μl of cDNA previously obtained by reverse transcription reaction and Master Mix reagents (Promega, Madison, WI) was used. Heparanase was amplified using forward (5′-CCCGAATTCAAAAAGTTCAAGAACAGCACC-3′) and reverse (5′-CCATGGTCAAGTGCAAGCAGCAACTTTGGC-3′) primers, and the product obtained was 1284 bp. PCR was run for 95°C for 10 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The primers 5′-AACGGGAAGCTCACTGGCATG-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTAG-3′ (reverse) were used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which yielded a product of 378 bp. PCR conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Products amplified by RT-PCR were analyzed by agarose gel electrophoresis, and each cDNA band was quantified by densitometry using the ImageTool analysis program (University of Texas Health Science Center in San Antonio, San Antonio, TX).

MCF-7 Cells

An established human breast cancer cell line (MCF-7) was cultured using Dulbecco's medium Eagle's medium (Life Technologies, St. Louis, MO) with added 1.2 g/l $NaHCO_3$, 0.1 g/ml streptomycin, 0.025 g/ml penicillin, and 10% fetal bovine serum in a humidified incubator at 37°C in the presence of 5% CO_2.

Immunocytochemistry

All antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). For immunostaining, we used an avidin–biotin–peroxidase complex and 3,3′-diaminobenzidine as chromogen. The following dilutions were used for each antibody: HPA1 C-20 (anti–heparanase 1) and HPA2 C-17 (anti–heparanase 2), 1:100; PC-10 (anti-PCNA), 1:1000; C-20 (anti-hMLH1), 1:25; K-20 (anti-hPMS1), 1:25; C-20 (anti-hPMS2), 1:200; N-20 (anti-hMSH2), 1:200; Pab1801 (anti-P53), 1:400. The preparation of immunocytochemistry slides has been described in detail by Fonseca et al. [22]. Two independent observers scored 300 cells/slide as positive or negative according to the presence of staining for each of the abovementioned antibodies. The results from these two observers were averaged to obtain the percentages of positive cells per sample.

Stimulatory Heparanase Assay in the Mononuclear Fraction of Peripheral Blood Samples

Lymphocyte fractions from healthy women were incubated with plasma or serum from breast cancer patients or with conditioned medium collected from MCF-7 cells for 4 hours at room temperature. In a coculture assay, MCF-7

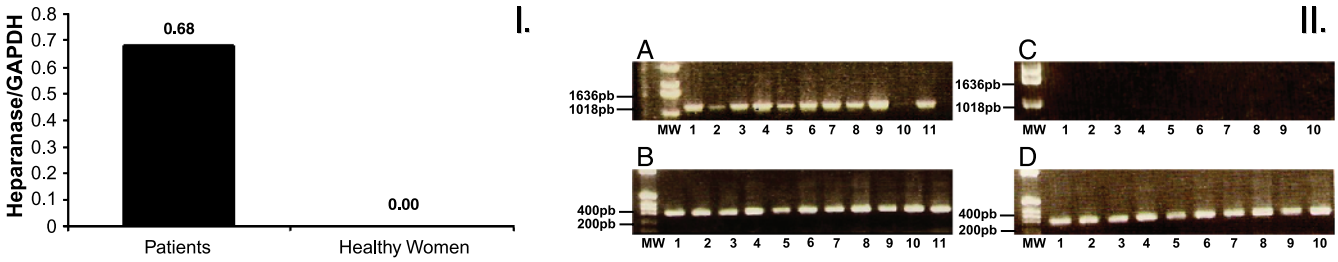

Figure 1. *Heparanase expression in the mononuclear fraction of peripheral blood samples. (I) Semiquantitative RT-PCR heparanase expression in the mononuclear fraction of breast cancer patients and healthy women. (II) Agarose gel electrophoresis: heparanase (A and C) and GAPDH (B and D). (A and B) Different samples from patients IMMP (1–6) and MBD (7–11). (C and D) Different samples from healthy women. Each sample was collected at 3-month intervals.*

cells were preincubated overnight with mononuclear fraction from a healthy woman's peripheral blood sample, and the conditioned medium collected was then incubated for 4 hours at room temperature with another mononuclear fraction from a healthy woman. It is important to point out that PBMCs used during the preincubation of coculture assays with MCF-7 cells were depleted of monocytes due to the preliminary differentiation of this cellular fraction using 40 nM phorbol 12-myristate 13-acetate (PMA) during 8 hours of incubation. This procedure of PBMC incubation using PMA promoted the differentiation of monocytes into macrophages, which became attached to culture plates, whereas lymphocytes were recovered from the supernatant and used for coculture incubation with MCF-7 cells overnight. Control assays were performed by the incubation of a healthy woman's PBMCs with a sample from the mononuclear fraction of another healthy woman.

Statistical Analysis

Statistical analysis was performed with the SPSS 13.0 program for Windows (SPSS, Chicago, IL). Variables in the study were considered parametric using the Kolmogorov-Smirnov test. Therefore, *t*-tests and paired *t*-tests were used.

Results

Serial Semiquantitative Heparanase RT-PCR in Peripheral Blood: A Marker for Breast Cancer Disease and Recurrence

In the present study, 30 patients with breast cancer and a mean age of 52 years (range, 25–80) were enrolled. Twelve patients had stage II breast cancer, 13 patients had stage III breast cancer, and 5 patients had stage IV breast cancer. The 20 normal control samples had a mean age of 50 years (range, 22–70). The expression of heparanase was present in PBMC samples analyzed from all 30 breast cancer patients (133.44 ± 53.44), whereas no expression was observed in the mononuclear fraction of 20 healthy women (Figure 1). Interestingly, heparanase expression detected by semiquantitative RT-PCR varied throughout the follow-up period of breast cancer patients (Figure 1), whereas no change in expression was observed in the samples of control healthy women collected at 3-month intervals (Figure 1).

When we attempted to correlate the variations of heparanase expression in positive PBMCs of breast cancer patients in relation to their clinical characteristics, we observed that: 1) heparanase expression decreased significantly after surgery ($P = .002$) in patients who underwent surgical removal of their tumors after neoadjuvant chemotherapy (Table 1); 2) heparanase expression was significantly higher in the PBMCs of metastatic patients receiving palliative treatment than in those with early breast cancer postsurgery ($P = .011$) (Table 1); and 3) heparanase expression increased significantly with the appearance of systemic metastasis (81.01 ± 17.01 before the metastatic event *vs* 142.90 ± 59.71 after the diagnosis of metastasis; $P = .027$) (Table 1).

Taken together, the above observations pointed toward the possibility that the presence of breast cancer tumor was associated with heparanase overexpression in PBMCs. We also observed a significant decrease in heparanase expression during tamoxifen treatment compared to chemotherapy or radiation therapy ($P = .040$) (Table 1).

Identification of Cells Expressing Heparanase in the PBMC Fraction By Immunocytochemistry

To ascertain which of the cells of PBMCs expressed heparanase, we used immunocytochemistry and polyclonal antibody anti-heparanase (HPA1 C-20). It can be observed in Figure 2 that lymphocytes from healthy women did not express heparanase 1, whereas breast cancer patients' lymphocytes expressed > 95% of heparanase 1 (HPA1). These data

Table 1. Clinical Characteristics and Heparanase Expression Measured By RT-PCR in the Mononuclear Fraction of Breast Cancer Patients.

Event	Heparanase Expression	*P*
Surgery		
Before	169.21 ± 45.40	.002
After	104.73 ± 46.25	
Adjuvant-versus-palliative treatment		
Free from disease	91.95 ± 54.61	.011
Palliative treatment	155.53 ± 45.58	
Metastasis or recurrence		
Before	81.01 ± 17.01	.027
After	142.90 ± 59.71	
Type of treatment		
Chemotherapy or radiotherapy	136.28 ± 57.32	.040
Tamoxifen	78.82 ± 54.20	

Numbers indicate the mean of heparanase expression and standard deviation. *P* indicates a comparison of means between patients' initial sample values and healthy women's heparanase expression.

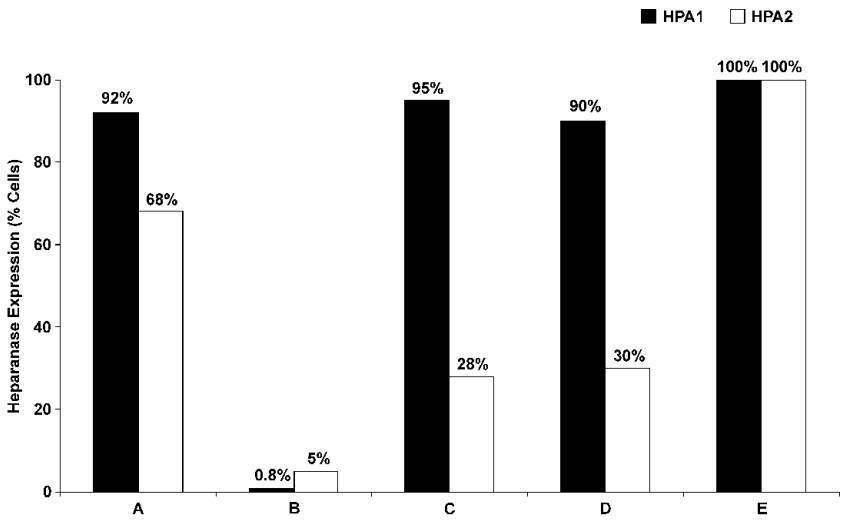

Figure 2. *Stimulatory effect of heparanase expression in PBMCs. Analyses were performed by immunocytochemistry using the antibodies anti-heparanase (HPA1 C-20; black) and anti–heparanase 2 (HPA2 C-17; white). Both antibodies were purchased from Santa Cruz Biotechnology, as described in Patients and Methods. (A) Breast cancer patients' mononuclear fraction cells (PBMCs). (B) A healthy woman's PBMCs. (C) A healthy woman incubated with plasma from a breast cancer patient. (D) A healthy woman's PBMCs incubated with MCF-7 cells. (E) MCF-7 cells.*

confirmed previous results obtained by semiquantitative RT-PCR where heparanase expression was analyzed by specific HPA1 primers.

We also investigated the expression of heparanase 2 (HPA2) in PBMCs using HPA2 C-17; interestingly, compared with the results obtained for HPA1, similar results were obtained. It was shown that 72% of breast cancer patients' lymphocytes were positive for heparanase 2 polyclonal antibody (HPA2 C-17), whereas only 8% of healthy women's lymphocytes were stained using the same technique (Figure 2).

Costimulation Experiments with Healthy Women's Lymphocytes

It is important to point out that the expression of heparanases HPA1 and HPA2 in the lymphocyte fraction of PBMCs was analyzed by immunocytochemistry, as described in Patients and Methods, and experimental conditions were derived from time-dependent and temperature-dependent curves to define the ideal condition for the incubation of mononuclear fraction lymphocytes to standardize the experiments (data not shown). Initially, we observed that both MCF-7 breast cancer cells and plasma from breast cancer patients were independently able to stimulate the expression of both heparanases in the lymphocytes of PBMCs from a healthy woman's mononuclear fraction cells incubated for 4 hours at room temperature (Figure 2). In contrast, no increase in heparanase expression was observed when the mononuclear fraction of a healthy woman's lymphocytes was incubated with another sample of PBMCs or with plasma from another healthy woman, showing that the observed activation of heparanase expression shown in previous experiments could not be related to exposure to a different individual plasma or PBMC preparation. Furthermore, similar experiments using sera from patients with breast cancer also induced an overexpression of both heparanases by PBMC lymphocytes from healthy women (data not shown).

Coculture of MCF-7 Cells and Lymphocytes from Healthy Women

Because MCF-7 cells in contact with a healthy woman's PBMCs induced an increase in HPA1 and HPA2 expression, the conditioned medium from MCF-7 cells was added to a healthy woman's PBMCs. The conditioned medium from MCF-7 cells was not able to stimulate a healthy woman's PBMCs when incubated for 4 hours at room temperature (Figure 3).

To evaluate whether cells from PBMCs would intermediate the effects of tumoral cells on the overexpression of both heparanases by normal lymphocytes, a new assay was developed. This assay involved initially coculturing MCF-7 cells with PBMCs from a healthy woman (18 hours, at 37°C, 5% CO_2). Then the conditioned medium collected from this coculture was assayed with another healthy woman's PBMCs for 4 hours at room temperature (Figure 3). Interestingly, the results have shown that the medium obtained from the coculture of MCF-7 cells and a healthy woman's PBMCs was able to stimulate HPA1 and HPA2 expression in the lymphocytes of another sample of PBMCs from a healthy woman (Figure 3).

We attempted to evaluate whether monocytes from PBMCs were the cells responsible for enriching the medium in the previous coculture in such a way as to lead to the increase in heparanase expression. Monocytes were removed from the PBMC fraction through their adherence to the surface of culture plates after treatment with PMA, and it was observed that there was still HPA1 and HPA2 overexpression induction mediated by the coculture of MCF-7 cells and PBMC fraction from a normal subject who was now poor in monocytes (data not shown).

Immunocytochemistry Analysis of PBMCs Using Other Tumor Markers

Finally, we decided to evaluate whether changes in the gene expression of PBMCs would be restricted to hepa-

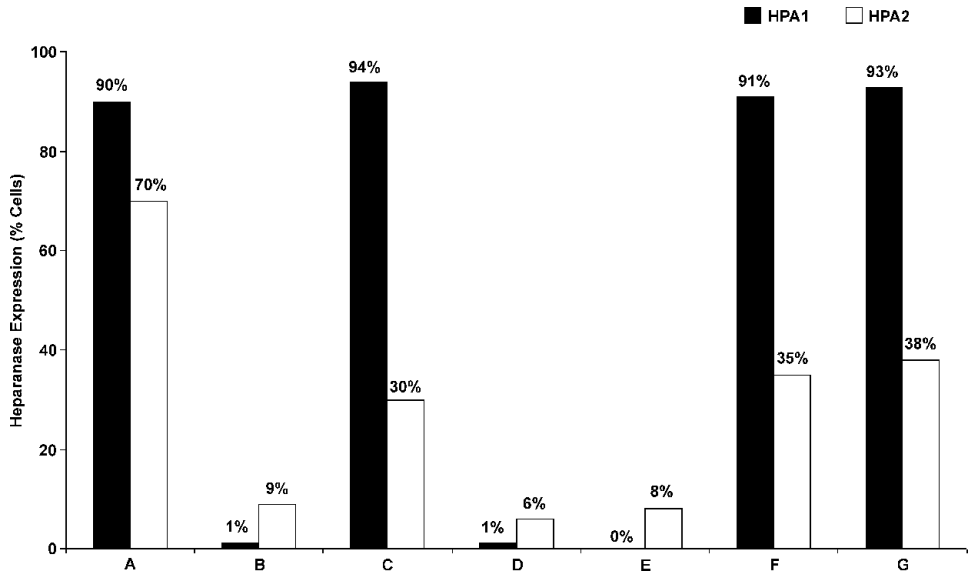

Figure 3. *Stimulatory effect of heparanase expression by a coculture assay in PBMCs. Analyses were performed by immunocytochemistry using the antibodies anti-heparanase (HPA1 C-20; black) and anti–heparanase 2 (HPA2 C-17; white), as described in Patients and Methods. Mononuclear fraction cells (PBMCs) from (A) a breast cancer patient; (B) a healthy woman; (C) a healthy woman incubated with plasma from a breast cancer patient; (D) a healthy woman incubated with the PBMCs of another sample from a healthy woman; (E) a healthy woman incubated with MCF-7 conditioned medium; (F) a healthy woman incubated with MCF-7 cells for 18 hours (coculture assay); and (G) a healthy woman incubated with coculture conditioned medium.*

ranases or could also happen for other proteins that are also important for tumor development. Thus, PBMC samples from the same breast cancer patients and from healthy women previously evaluated for heparanase expression were then investigated for PCNA, P53, hMLH1, hPMS1, hPMS2, and hMSH2. We observed significant differences in the expression of PCNA ($P = .031$), hMSH2 ($P = .018$), and hPMSH2 ($P = .017$) between healthy women and breast cancer patients (Table 2). No significant differences were observed in the expression of the aforementioned proteins between the initial samples and the repeated samples of the same healthy women collected 3 months later (Table 2).

Discussion

It is important to point out that the gene encoding HPA1 maps to human chromosome 4q21.3, whereas human chromosome 10q23–24 encodes three alternative splice variants of HPA2. Despite being encoded by different chromosomes, the expression of both heparanases in the lymphocytes of PBMCs of healthy women was stimulated by plasma, serum, or tumor cells. These results could suggest that possibly heparanase isoforms present at least one mechanism of gene control in common.

Our data further suggest that this tumor-inducing effect on both HPA1 and HPA2 expression is not directly mediated by tumor cells, but depends on the presence of nonneoplastic cells in PBMCs that seem to be lymphocytes and not monocytes. Additionally, tumor-inducing effects on the gene expression of PBMCs are not restricted to heparanases, but can also be shown in genes related to the DNA repair mechanism, such as hMSH2 and hPMS2, and in cell proliferation–associated genes, such as PCNA. Taken together, our data also suggest that a tumor-induced systemic effect, produced through an interaction between tumor cells and cells present in PBMCs, affects the expression of several genes from nontumoral cells. Conceivably, these effects could contribute to enhanced primary tumor growth, metastasis, and angiogenesis.

In vivo, it is possible that the tumor may induce its infiltrating lymphocytes (TILs) to produce a factor that, in the systemic circulation, may in turn lead to changes in gene

Table 2. Quantification of Different Proteins Related to Cell Proliferation, DNA Repair, and Apoptosis by Immunocytochemistry Assay.

Immunohistochemistry Reaction	NC T_0 ($n = 20$)	NC T_3 ($n = 20$)	Patients ($n = 28$)	P
PCNA	15.93 ± 4.57	17.20 ± 4.57	31.70 ± 3.86	.031
hMSH2	72.70 ± 4.50	75.81 ± 4.50	56.22 ± 3.80	.018
hMLH1	0 ± 0.21	0 ± 0.21	0.60 ± 0.18	.080
hPMS1	0.60 ± 0.33	0.10 ± 0.33	0.73 ± 0.28	.780
P53	ND	ND	ND	
hPMSH2	0 ± 3.33	0 ± 3.33	12.84 ± 2.81	.017

Numbers represent the mean ± standard deviation of the percentage of positive cells for PCNA, hMSH2, hMLH1, hPMS1, P53, and hPMSH2.
NC = normal controls; T_0 = samples obtained initially; T_3 = samples obtained after a 3-month interval; SD = standard deviation; N = number of patients; ND = not detected.
P values reflect a comparisons of means between patients' initial samples and normal controls' first samples.

expression in various non-neoplastic cells and potentially also in malignant cells because these changes in gene expression can be induced by treatment with conditioned media from cocultures of MCF-7 and non-neoplastic PBMCs. Furthermore, because the expression of heparanase decreases in patients with breast cancer rendered free of tumor by surgery, it is possible that these changes in heparanase gene expression depend on the dysregulation of gene expression by reversible mechanisms such as promoter methylation and promoter inhibition by P53 binding [23,24].

In line with our results, Kataoka et al. [25] showed that media from cultured rabbit carcinoma cells stimulated the secretion of interstitial collagenase by fibroblasts. Dabbous et al. [26,27] also demonstrated that fibroblast-like and endothelial-like cells derived from carcinoma cultures released more interstitial collagenase than normal fibroblasts. In addition, in accordance with this complexity of normal–tumor cell interactions, Henry et al. [28] showed that cocultures of mouse peritoneal macrophages and Lewis lung carcinoma cells induce the release of a type IV collagen–degrading metalloproteinase activity and proteoglycan-degrading enzymes that are not secreted in detectable amounts by either cell type alone. Dabbous et al. [26,27] also reported that tumor-associated mast cells stimulated fibroblasts to secrete interstitial collagenase activity.

It is known that inflammation plays a critical role in cancer progression, and some studies have demonstrated that macrophage interaction with cancer cells promotes a protumorigenic activity that changes the gene expression profile in lung cancer cells [28,29]. In our study, however, our data suggest that it is the interaction between tumor cells and lymphocytes that seems to be important for heparanase expression induction by the PBMC fraction in cancer patients.

Interestingly, our data show a significant difference in heparanase expression in the mononuclear fraction of peripheral blood samples from breast cancer patients without evidence of malignancy compared to those from patients with known active disease and from healthy women. Therefore, heparanase expression in the PBMCs of breast cancer patients may be also a marker of disease activity, and future studies should be conducted to evaluate this interesting hypothesis.

We also observed a significant decrease in heparanase expression in PBMCs from patients submitted to tamoxifen treatment, in comparison to chemotherapy or radiotherapy. This result could be due to the fact that tamoxifen competes with estrogen for estrogen receptors that, in turn, when activated by estrogen, increase heparanase expression by directly activating the heparanase promoter [30,31]. So far, our data have agreed with what has been shown in *in vitro* assays using culture cells [30].

As heparanase expression seems to be due to its overregulation by normal cells, we do not believe that detection of heparanase-expressing cells in PBMC fractions necessarily indicates the presence of circulating malignant cells for which detection would be useful for minimal residual disease assessment in these patients. Nevertheless, we cannot exclude that heparanase overregulation in PBMC fractions may itself be a tumor marker. Further studies are necessary, however, to confirm this contention.

These studies have demonstrated a possible mechanism of interaction between tumor cells with PBMCs that activate heparanase expression by non-neoplastic cells, as well as other proteins. Whether the cells of PBMCs are only bystanders of the systemic activation of some of these changes in gene expression or whether they could be important for tumor progression is unknown at present. A better characterization of these systemic effects resulting from tumor–normal cell interactions may yield new tumor markers and may allow us to delineate new treatment strategies for women with breast cancer.

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