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ORIGINAL PAPER

Analysis of the expression of hyaluronan in intraductal and invasive carcinomas of the breast

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Abstract
Aims To evaluate hyaluronan expression at different stages of tumoral progression in primary breast cancer.
Methods Hyaluronan expression was evaluated by histochemical techniques in 42 cases of pure DCIS, in 15 cases of DCIS with a microinvasive component, and in 32 cases of invasive ductal carcinoma of the breast. Staining results were evaluated by calculating the percentage of stained areas by means of a specific software program.
Results Our results show higher values of hyaluronan expression in invasive breast carcinomas [median of percentage of stained areas 41.1 (range 8–69.2)] and in DCIS with a microinvasive component [48.6 (16.8–62.8)] than in pure DCIS [14.5 (1–44.4)] ($p < 0.001$, for both).
Conclusions Our study indicates a proportionally higher area of hyaluronan expression in DCIS with a microinvasive component than in pure DCIS, suggesting a key role of this glycosaminoglycan in the early invasive phase of breast carcinomas. Thus, hyaluronan could play an important function in determining the migratory phenotype of cancer cells. Larger size tumors appear to demonstrate an intricate balance between hyaluronan synthesis and degradation, thus conditioning intratumoral heterogeneity in the hyaluronan metabolism.

Keywords Breast cancer · Hyaluronan · CD44 · Tumoral invasion

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Introduction

Hyaluronan is a high molecular weight acidic polysaccharide found on the cell surface and in the extracellular matrix of most human tissues (Fraser et al. 1997; Prehm 1984). It is synthesized at the plasma membrane level by the enzyme hyaluronan synthase and is then extruded, while still elongating into the extracellular matrix (Prehm 1984). Its expression is increased during physiologic tissue remodeling processes characterized by rapid cell proliferation, as in wound healing and morphogenesis (Toole 1990). As a component of extracellular matrices, hyaluronan influences tissue morphogenesis and has several physiological functions, such as water homeostasis, regulation of capillary growth, cell recognition, and cell migration (Fraser et al. 1997). In addition, elevated concentrations of hyaluronan have also been found in several human tumors (Arai et al. 1979; Hopwood and Dorfman 1978; Llaneza et al. 2000; Setala et al. 1999; Sowa et al. 1989; Vizoso et al. 2004; Wang et al. 1996), including breast carcinomas (Auvinen et al. 2000; Bertrand et al. 1992; de la Torre et al. 1993; Ponting et al. 1992; Takeuchi et al. 1976). Likewise, experimental

studies have shown that increased concentrations of hyaluronan stimulate cell motility (Goebeler et al. 1996), cell adhesion (Catterall et al. 1999), neovascularization (Koyama et al. 2007; Rooney et al. 1995) and development of metastases in neoplastic tissues (Zhang et al. 1995). Cells bind to hyaluronan through at least two cell-surface receptor proteins, CD44 and receptor hyaluronan-mediated motility (RHAMM) (Rudzki and Jothy 1997).

Despite all of the above mentioned evidence pointing towards a key role of hyaluronan in the regulation of the steps of tumor growth, tissue invasion and metastases, there is scarce information on its clinical significance in breast cancer. Up to now, only two studies have determined, by histochemical methods, that elevated hyaluronan expression in breast tumors is associated with clinicopathological parameters indicative of tumor aggressiveness, such as positive nodes and high tumor grade (Auvinen et al. 2000; Suwiwat et al. 2004; Wernicke et al. 2003), as well as a shorter overall survival (Auvinen et al. 2000). However, in the only published work analyzing levels of high molecular weight hyaluronan (HMW-HA) by immunoradiometric assays, we found a significant and positive relationship between high intratumoral levels of hyaluronan and classic clinicopathological parameters indicative of lower tumor aggressiveness, such as a high histological grade of differentiation, diploid tumors and both estrogen (ER) and progesterone receptor (PR) positive tumors. Consistent with these findings, we also determined that high HMW-HA levels were significantly associated with a longer relapse-free survival in the subgroup of patients with tumors of the ductal histological type, even among those with node-negative tumors, and also when we considered different subgroups of patients with regard to the adjuvant systemic therapy received (Corte et al. 2006).

In order to gain a better understanding of the role of hyaluronan in different phases of tumor progression, we investigated the possible differential expression of hyaluronan in DCIS of the breast, DCIS with a microinvasive component, and invasive ductal carcinomas of the breast.

Materials and methods

Patient characteristics and tissue specimen handling

Formalin-fixed, paraffin-embedded breast tissue sections were obtained from a total of 86 patients: 42 consecutive patients with the diagnosis of pure DCIS, 15 consecutive patients with DCIS with a microinvasive component, and 32 consecutive patients with invasive ductal carcinoma of the breast. Patient and tumor characteristics are listed in Tables 1 and 2. Tissue samples were obtained at the time of surgery at Fundación Hospital de Jove of Gijón (Spain), between 1995 and 2002.

Table 1 Relationship between histochemical staining and clinicopathological characteristics in 42 cases of DCIS of the breast

Clinicopathological and biological characteristics	Hyaluronan morphometric expression			
	N	Median	Range	*p*
Total cases	42	14.5	1–44.4	
Age (years)				NS
≤56	21	19.4	1.2–44.4	
>56	21	12.7	1–29.6	
Menopausal status				NS
Premenopausal	12	18.9	1.2–44.4	
Postmenopausal	30	13.6	1–33.9	
Architectural subtypes				NS
Comedo	7	2	1–29.8	
Cribiform	6	11	1–42.5	
Micropapillary	4	16	2.5–21.4	
Mixed	13	20.6	2.9–39.5	
Solid	12	16.4	3.6–44.4	
Histologic grade				NS
G-1	16	15.5	1–42.5	
G-2	5	12.4	1.6–26.9	
G-3	21	15.2	1–44.4	
Estrogen receptors				NS
Negative	12	11.2	1.2–29.6	
Positive	30	16.4	1–44.4	
Progesterone receptors				0.03
Negative	17	11.0	1–29.6	
Positive	25	17.4	1.6–44.4	

Patients with DCIS were normally treated by either quadrantectomy or segmental resection, and the corresponding archived histological slides were reviewed and reclassified according to the Consensus Conference of 1997 (1997). Microinvasive carcinoma was defined as a tumor in which the dominant lesion was non-invasive but with one or more clearly separated foci of infiltration none of which measured more than 1 mm in diameter (1995).

Patients with invasive breast cancer were women without distant metastases at the time of the study. They underwent either a partial or a total mastectomy with axillary lymph node dissection. None of the patients had received neoadjuvant therapy nor shown evidence of any other malignant tumors at the time of diagnosis. Histological grade was determined according to the criteria reported by Elston and Ellis (1993), whereas nodal status was assessed histopathologically.

Tissue samples from tumors were obtained with prior informed consent of the patients, and the study adhered to national regulations and ethical issues, being approved by our institution's Ethics and Investigation Committee.

Table 2 Relationship between hyaluronan immune morphometric staining and clinicopathological characteristics in 32 cases of ductal invasive breast carcinomas

Patient and tumor characteristics	Hyaluronan morphometric expression			
	N	Median	Range	*p*
Total	32	41.1	8–69.2	
Age (year)				NS
<54 years	10	32.2	20.5–61.9	
>54 years	22	41.7	8–69.2	
Menopausal status				NS
Premenopausal	10	32.2	20.5–55	
Postmenopausal	22	41.7	8–69.2	
Size				NS
T1	9	38.9	8–63.9	
T2	19	44.5	15.3–69.2	
T3	3	26.7	20.5–31.3	
T4	1	39.2	39.2–39.2	
Nodal status				NS
N(−)	22	41.1	8–69.2	
N(+)	10	37	15.3–61.9	
Histological grade				
Well Dif	13	40	8–63.9	
Mod. Dif.	9	49	26.7–69.2	
Poorly Dif.	10	41.1	26.4–61.9	
Estrogen receptors				NS
Negative	10	42.8	26.7–61.9	
Positive	22	40	8–69.2	
Progesterone receptors				NS
Negative	17	41	8–69.2	
Positive	15	42.3	20.5–63.9	

Quantitative morphometry of hyaluronan-stained stromal signals

Histochemical assays were performed on 5-μm, formalin-fixed, paraffin-embedded tissue sections using the avidin–biotin system and the peroxidase/DAB detection kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions. Tissue sections were deparaffinized in xylene, and then rehydrated in graded concentrations of ethyl alcohol (100, 96, 80, 70%, then water). A Dako TechMate TM50 autostainer (Dako) was used to stain the samples. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako ChemMate TM) for 5 min. Five micrometer-thick sections were incubated with biotinylated cartilage HA-binding protein (Calbiochem, USA) for 30 min.

Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently cover slipped. The staining specificity was determined using controls that involved incubation of tissue sections with antibody diluent (Dako) alone. In addition, we used negative controls by digestion of the sections with *Streptomyces* hyaluronidase.

An image analysis system using an Olympus BX51 microscope and software analysis (AnalySIS®, Soft Imaging System, Münster, Germany) was utilized as follows: tumor sections were stained according to the method described above and counterstained with haematoxylin. There were different optical thresholds for each stain. Each slide was scanned with a 400 × power objective. Fields were selected by searching for the protein-stained areas. The computer program selected and traced a line around areas stained with the HA-binding protein (higher optical threshold, red spots), with the remaining, non-stained areas (haematoxylin-stained tissue with lower optical threshold) standing out as a blue background. Each field had a percentage histochemical staining area. The area measured included both cancer cells and the stroma. A final percentage was obtained after averaging ten different 2-mm diameter fields.

Statistical analysis

After analyzing the tumor distribution of hyaluronan values by the Kolmogorov–Smirnov test, non-parametric methods were used to analyze the data. Hyaluronan content was expressed as a median (range). Patients were subdivided into groups based on different clinical and pathological parameters. Comparison of HA content among groups was made with the non-parametric Mann–Whitney and Kruskal–Wallis tests.

However, while the mean values of staining intensity (1+, 2+, or 3+) for hyaluronan showed normal distributions, these data were expressed as mean ± standard deviation (SD) and compared with the ANOVA test. The SPSS 11.5 program was used for all calculations. Statistical significance was considered at the 5% probability level ($p = 0.05$).

Results

Hyaluronan expression was evaluated by histochemical techniques in 42 cases of pure DCIS, 15 cases of DCIS with a microinvasive component, and 32 cases of invasive ductal carcinoma of the breast. Hyaluronan-positive staining was localized in the stroma adjacent to DCIS and in the peritumoral and/or intratumoral stroma of the invasive tumors. Representative examples of positively stained tissue sections are shown in Fig. 1. We also investigated the presence of hyaluronan-positive staining in tumor cells. None of the 42 DCIS included in the present study showed hyaluronan-positive staining in their tumor cells, whereas it was present in 4 of the 15 (26%) DCIS with a microinvasive component, and in 11 cases of the 32 (34.4%) invasive ductal carcinomas

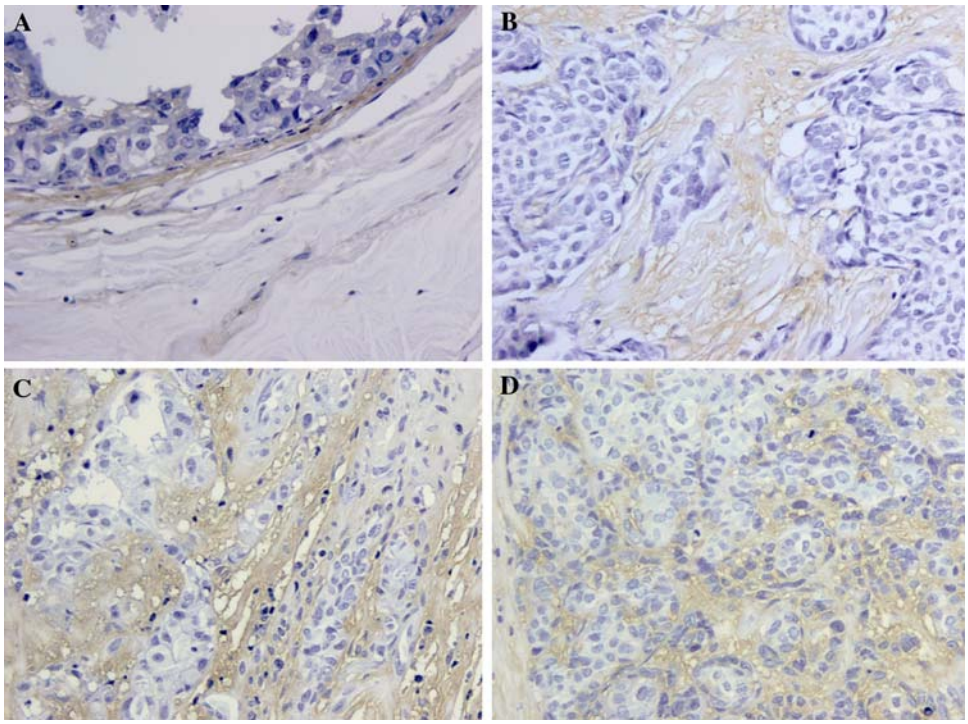

Fig. 1 Representative examples of histochemical staining for hyaluronan of tumors ×200. **a** DCIS, **b** DCIS with microinvasive component. **c** and **d** Ductal invasive carcinoma

of the breast. However, we did not find any significant association between hyaluronan-positive staining in tumor cells and the clinicopathological or biological characteristics of the tumors (data not shown).

We analyzed the mean value of staining intensity (1+, 2+, or 3+) for hyaluronan of ten different 2-mm diameters areas in each tumor. Our results showed a significantly higher value of the mean staining intensity for hyaluronan in areas of both invasive breast carcinomas [mean ± SD, 2.2 ± 0.23 (range 1.9–3)] and DCIS with microinvasive foci [(mean ± SD, 2.17 ± 0.45 (range 1.8–3.3)] than in pure DCIS [(mean ± SD, 1.58 ± 0.12 (range 1.4–1.7) ($p < 0.0001$)]. Our results show high percentage of stained area for the expression of hyaluronan in invasive breast carcinomas [median of the percentage of stained area, 41.1 (range 8–69.2)] and in DCIS with a microinvasive component [median 48.6 (range 16.8–62.8)], as compared to pure DCIS [median 14 (range 1–44)] ($p < 0.001$, for both) (Fig. 2). It was of note that although the area measured included both cancer cells and the stroma, the influence of the stained area corresponding to cancer cells was minimal and without to influence significantly these latter results. Nevertheless, considering that invasive carcinomas contain more cancer cells than DCIS, we also analyze stromal tissue removing cancer cells in these tumors. The results showed a very similar percentage of hyaluronan-positive area in the overall neoplastic tissue compared with the one in the single stromal component in invasive breast tumors [median (range); 16.5 (4.5–29.2) vs. 17.5 (3.8–29.1), respectively].

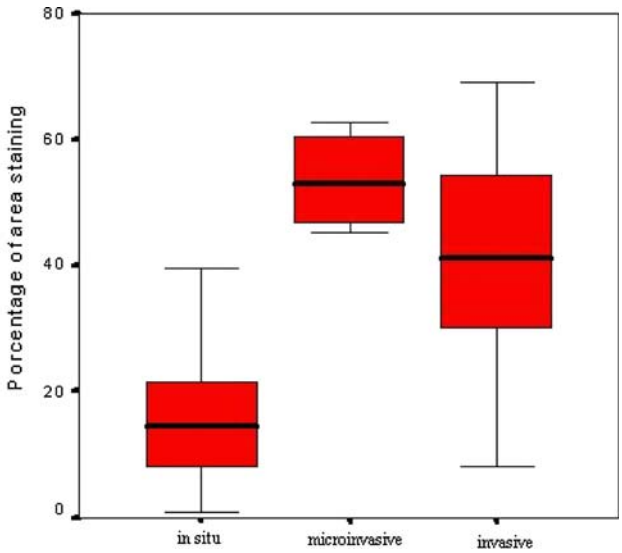

Fig. 2 Staining values for hyaluronan in 42 DCIS, in 15 DCIS with microinvasive component, and in 32 ductal invasive carcinomas of the breast

The relationships between histochemical staining for hyaluronan and the clinicopathological and biological characteristics of the tumors are shown in Table 1. The clinicopathological characteristics evaluated for DCIS tumors included: patient age and menopausal status, the architectural subtype and histological grade, according to Van Nuys' classification, as well as the ER and PR status; whereas for invasive breast carcinomas the characteristics studied were: age, menopausal status, tumor size, axillary

node involvement, histological type, histological grade, and ER and PR status (Table 2). There was a positive and significant relationship between histochemical expression of hyaluronan and PR in pure DCIS (Table 1). However, we did not find any significant relationship between hyaluronan expression and the clinicopathological characteristics evaluated in invasive tumors (Table 2).

Discussion

The present study demonstrates a different hyaluronan expression in the various phases of tumor progression in primary breast cancer. Thus, we found that hyaluronan expression is located in the microinvasive foci of DCIS, whereas the stroma adjacent to pure DCIS is only faintly positive. This study demonstrates that hyaluronan expression is prominent during the initial injury of the extracellular matrix by breast cancer cells. On the other hand, we found wide intra- and inter-tumoral heterogeneity of hyaluronan expression in invasive breast carcinomas of larger size.

The highest area of hyaluronan expression in the microinvasive component of DCIS might be due to a major interaction of the malignant epithelial cells with the stromal components. Hyaluronan is one of the major stromal constituents, and it is known that its synthesis is stimulated by the interaction between tumor and stromal cells (Brown et al. 1995; Knudson et al. 1984). Thus, the high level of expression of the glycosaminoglycan may be one of the first steps in tumoral invasion. Accordingly, recent experimental studies suggest a central role of hyaluronan synthases in the initiation and progression of breast cancer (Udabage et al. 2005a, b). The effects of hyaluronan may be based on its physicochemical properties, and on the migratory and mitogenic signals mediated through its cell-surface receptors, such as CD44 and RHAMM (Entwistle et al. 1996). First, the presence of the highly hydrophilic hyaluronan in the intercellular space between malignant cells contributes to binding water and, consequently, tissue expansion. This allows hyaluronan to create and fill the extracellular space, thus regulating cell movement and transport of extracellular components (Toole 2004). Secondly, it has been demonstrated that the high molecular mass hyaluronan acts as a soluble chemoattractant promoting the directional migration of breast cancer cells, which is dependent on CD44 expression and is modulated by the cell type variation in CD44-hyaluronan binding (Tzircotis et al. 2005). These findings suggest that there is a direct mechanism for promoting cell infiltration into the surrounding matrix, particularly where large accumulations of hyaluronan occur in the tumor-associated stroma. All these observations, derived from experimental studies, support the notion of a relevant role of hyaluronan, at least in some steps in the course of tumoral progression.

Histochemical studies have reported the expression of hyaluronan as being associated with poor prognosis in some groups of patients with breast carcinomas (Auvinen et al. 2000; Bertrand et al. 1992), whereas high hyaluronan levels determined by immunoradiometric assays have been associated with high ER or PR content, as well as a favorable outcome in patients with ductal invasive breast cancer (Corte et al. 2006). Similarly, Kosunen et al. found that a reduced expression of hyaluronan was a strong indicator of poor survival of patients with squamous cell carcinomas (Kosunen et al. 2004), but this is a totally different cancer type which shows a reduced hyaluronan expression in poorly differentiated advanced tumors. The discrepancy between these studies on breast cancer may be more apparent than real. It is possible that the most invasive tumors show less hyaluronan due to a higher degradation by hyaluronidases and other proteolytic enzymes. Thus, it is likely that ER-positive and/or PR-positive breast tumors are less aggressive and induce less hyaluronan degradation. In accordance with this, it is remarkable that in the present study we find a positive relationship between hyaluronan expression and PR status in pure DCIS, considering that PR is an estrogen-induced protein whose expression seems to indicate a functionally intact ER pathway.

In conclusion, our results indicate a high level of hyaluronan expression in DCIS with a microinvasive component, suggesting a key role of this glycosaminoglycan in the early invasive phase of breast carcinomas. Therefore, hyaluronan could play an important role in determining the migratory phenotype of cancer cells. However, in tumors of larger size there appears to be an intricate balance between both hyaluronan synthesis and degradation, conditioning intratumoral heterogeneity in the hyaluronan metabolism. Although in the present work only total hyaluronan levels were determined, we also consider the importance of further studies based on the different cellular responses in breast cancer to hyaluronan of distinct molecular sizes. In general, high molecular mass hyaluronan has been associated with a structural role (as a space-filling molecule, a component of the pericellular matrix and a scaffold for migrating cells), whereas it has been suggested that hyaluronan fragments are more biologically active with respect to stimulating signal transduction in tumor cells (Rooney et al. 1995; Sugahara et al. 2003).

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Conflict of interest statement We declare that we have no conflict of interest.

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