Hyaluronan Promotes Tumor Lymphangiogenesis and Intralymphantic Tumor Growth in Xenografts

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Abstract   Hyaluronan (HA), a high molecular weight glycosaminoglycan in the extracellular matrix, has been implicated in the promotion of malignant phenotypes, including tumor angiogenesis. However, little is known about the effect of HA on tumor-associated lymphangiogenesis. In this study, mouse hepatocellular carcinoma Hca-F cells combined with or without HA were injected subcutaneously into C3H/Hej mice, then angiogenesis and lymphangiogenesis of implanted tumors were examined by immunostaining for platelet-endothelial cell adhesion molecule-1 and lymphatic vascular endothelial hyaluronan receptor-1 respectively. Interestingly, we found HA promotes tumor lymphangiogenesis and the occurrence of intratumoral lymphatic vessels, but has little effect on tumor angiogenesis. Moreover, HA also promotes intralymphatic tumor growth, although it is not sufficient to potentiate lymphatic metastasis. These results suggest that HA, which is elevated in most malignant tumor stroma, may also play a role in tumor progression by promoting lymphangiogenesis.

Key words   hyaluronan; tumor lymphangiogenesis; intralymphatic tumor growth

Lymphangiogenesis, the formation of new lymphatics, is a fundamental physiological process required for the development of the embryonic lymph system and regeneration of lymphatic vessels in adults, for example, in wound healing [1]. More recently, lymphangiogenesis has also been implicated in tumor progression [2,3]. However, the regulation of tumor-associated lymphangiogenesis is not well understood. Animal models demonstrated that tumor-associated lymphangiogenesis can be induced by overexpression of lymphangiogenic vascular endothelial growth factor (VEGF)-C or VEGF-D, and that lymphangiogenesis may be involved in subsequent tumor lymphatic metastasis [4–6]. Moreover, targeting of VEGF-C/VEGF-D and their receptor VEGFR-3 signaling resulted in decreased tumor lymphangiogenesis and reduction of lymph node metastases [6–9]. These data suggested the important roles of VEGF-C, VEGF-D and their receptor VEGFR-3 in tumor lymphangiogenesis and lymph node metastasis.

Hyaluronan (HA) is a high molecular weight glycosaminoglycan in the extracellular matrix. HA has been shown to play important roles in tumor progression. Most malignant solid tumors contain elevated levels of HA, and in some cases HA levels were prognostic for malignant progression [10]; on the other hand, HA has been implicated in regulating tumor malignant behaviors, for example, anchorage-independent growth [10], tumor cell motility [11,12], secretion of matrix metalloproteinases [13], as well as tumor angiogenesis [14,15]. Despite the wealth of data, however, it has not yet been addressed whether HA has any effect on tumor-associated lymphangiogenesis.

Experimental manipulations of HA concentration in tumor stroma by overexpression of HA synthase or administration of exogenous HA have been used to investigate the role of HA in tumor progression [14,15].

In this study, we examined the effect of exogenous HA on tumor lymphangiogenesis, angiogenesis and lymphatic metastasis in a lymph node metastatic model. The data showed that HA promotes the formation of intratumoral lymphatic vessels and intralymphatic tumor growth. Our

results suggest that HA may be a potential new regulator in tumor lymphangiogenesis.

Materials and Methods

Cells

Mouse hepatocellular carcinoma Hca-F cells were injected i.p. into 8-week-old C3H/Hej mice at 1 ml cell solution per mouse (10^7 cells/ml PBS) to generate ascites. Ascites were then harvested and injected into new mice.

Tumorigenesis and tumor growth assay

Hca-F ascites were harvested and washed twice with PBS, then resuspended in PBS or 1 mg/ml HA (hyaluronic acid sodium salt from human umbilical cord; Fluka, St. Louis, USA), which were injected s.c. into the right flank region of 8-week-old C3H/Hej mice at 100 μl per mouse (2×10^6 cells/100 μl). Tumor length and width were measured, and the tumor volume (cm^3) was calculated as length$\times$width$^2\times$0.52.

Immunostaining for lymphangiogenesis and angiogenesis

The tumors were excised and frozen in liquid nitrogen. Sections (5 μm) were immunostained with rat monoclonal antibody against platelet-endothelial cell adhesion molecule-1 (PECAM-1) (PharMingen, Franklin Lakes, USA) [16] or rabbit polyclonal antibody against mouse lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1) (a kind gift from Dr. David G. JACKSON, Oxford University, UK) [17]. The staining was detected with an ABC kit (Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions. The slides were stained with diaminobenzidine (Huamei, Shanghai, China), washed, counterstained with hematoxylin, dehydrated with xylene and mounted. The numbers of PECAM-1$^+$ spots corresponding to small blood vessels were counted in three areas (100$\times$) of the highest vascular density (vascular hot spots) from five tumors of each group.

Tumor metastasis assay

Superficial axillary, deep axillary and inguinal lymph nodes (on right side) were removed, fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5 μm) were cut, stained with hematoxylin and eosin, and examined microscopically. The rate of metastasis was calculated as metastatic mice per total tested mice, and the rate of lymph node metastasis was calculated as the number of positive lymph nodes per total number of lymph nodes examined.

Statistical analysis

Data are presented as mean±SD. For statistical analysis, Student's *t*-test was performed. $P<0.05$ was considered to denote statistical significance.

Results

HA induced tumor lymphangiogenesis in xenografts

LYVE-1 is a highly specific molecular marker of lymphatic endothelia and has been widely used in the detection of lymphangiogenesis [17]. Here, we examined the effect of exogenous HA on lymphangiogenesis by immunostaining for LYVE-1 to show the lymphatic vessels in implanted tumors. Tumors were sectioned and subjected to immunostaining at day 21 after implantation. In control tumors, peritumoral lymphatic vessels are present along the boundary of the control tumor [**Fig. 1(A)**], and few intratumoral lymphatics can be detected [**Fig. 1(B)**]. Furthermore, inside the tumor body, there are only large tumor cells, and no LYVE-1 positive cells are found [**Fig. 1(C)**]. In contrast, intratumoral lymphatic vessels are present in the HA-treated tumors [**Fig. 1(D–F)**]. Those intratumoral lymphatic vessels appear heterogeneous, because most of them congregate together in clusters [**Fig. 1(D,E)**]. Some of them are close to the tumor periphery [**Fig. 1(D)**], and some are deep inside the area [**Fig. 1(E)**]. The intratumoral lymphatic vessels should be hyperplastic, because LYVE-1$^+$ endothelial cells are frequently detected at the higher magnification of 400$\times$. These cells are flat in shape and morphologically different from surrounding tumor cells [**Fig. 1(F)**]. The LYVE-1$^+$ cells congregate together, and the lymphatic vessel is taking shape [**Fig. 1 (F)**]. These results demonstrate that HA promotes the formation of lymphatic vessels in xenografts.
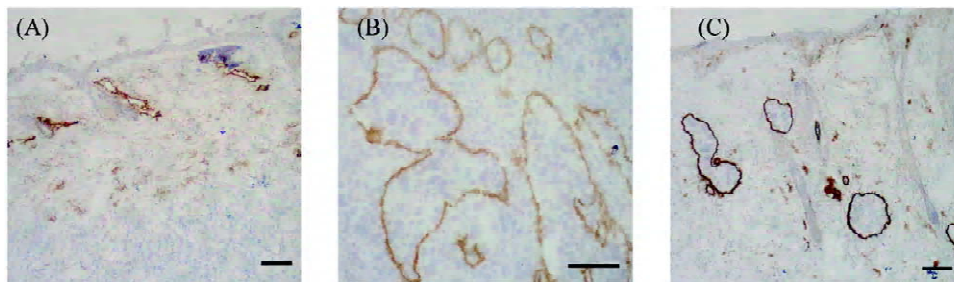
HA induced intralymphatic tumor growth

Besides its effect on lymphangiogenesis, we found it interesting that exogenous HA also promotes intralymphatic growth of tumor cells. In control tumors, few tumor cells were found in either peritumoral or subcutaneous lymphatic vessels [**Fig. 2(A)**]. However, in HA-treated tumors, it can be clearly observed that intratumoral lymphatic vessels are commonly infiltrated with tumor cells [**Fig. 2(B)**]. Furthermore, the tumor cells also grow in neighboring subcutaneous lymphatic vessels [**Fig. 2(C)**]. Both the
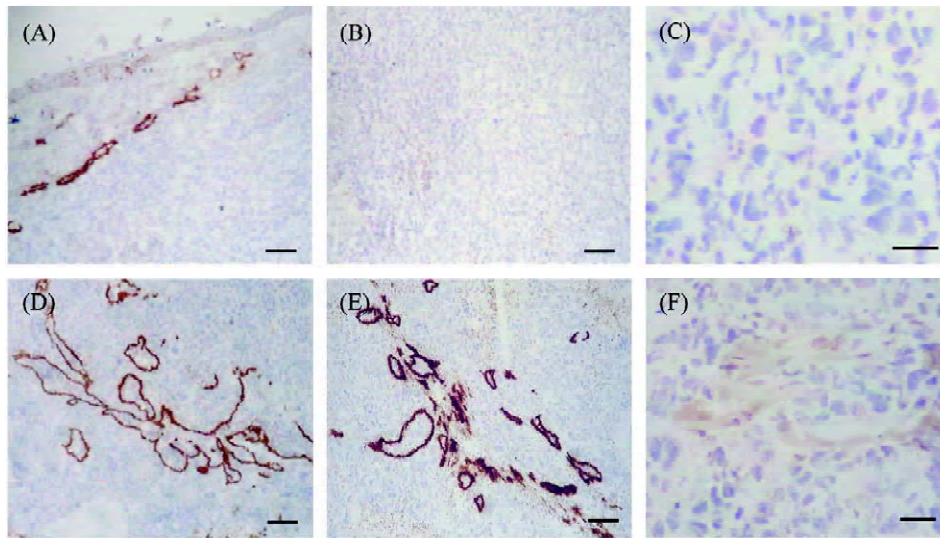
Fig. 1   Exogenous hyaluronan (HA) induced tumor lymphangiogenesis

In the control tumors (A–C), LYVE-1+ (brown) lymphatic vessels can be detected in the peritumoral area (A), but not in the intratumoral area (B); there are no LYVE-1+ cells inside the tumor body (C). In the HA-treated tumors (D–F), intratumoral lymphatic vessels can be detected near the tumor margin (D), or in the central region (E); LYVE-1+ cells congregate together, and the lymphatic vessel is coming into being (F). Bar=75 μm in (A), (B), (D) and (E); bar=25 μm in (C) and (F).

Fig. 2   Exogenous hyaluronan (HA) induced intralymphatic tumor growth

(A) Few tumor cells can be found in the peritumoral or subcutaneous lymphatic vessels in control tumors. Both intratumoral (B) and neighboring subcutaneous (C) lymphatic vessels are infiltrated with tumor emboli in HA-treated tumors. Bar=100 μm.

intratumoral and the subcutaneous lymphatic vessels filled with tumor cells are strikingly enlarged and display irregular morphology [**Fig. 2(B,C)**]. In general, HA promotes intralymphatic tumor growth.

Effect of exogenous HA on tumor angiogenesis and tumor growth

It has been reported that HA may be involved in the promotion of tumor angiogenesis [18]. We next examined whether HA has any effect on angiogenesis in our model. The blood vessels were detected by immunostaining the frozen sections of implanted tumors with PECAM-1 (CD31), a specific endothelial marker expressed mainly on blood vessels [16]. As shown in **Fig. 3(A)**, the mor-

phology of tumor blood vessels is apparently different from lymphatic vessels, because the former is significantly smaller than lymphatic vessels. The tumor angiogenesis was quantitatively assessed by counting the numbers of PECAM-1+ spots in three areas from five tumors of each group. It was shown that exogenous HA has little effect on tumor angiogenesis, as quantified by the density of the tumor blood vessels (220 ± 9 vessels/microscopic field for HA-treated tumors and 216 ± 11 vessels/microscopic field for control tumors) [**Fig. 3(B)**].

Previously, it was demonstrated that endogenous HA promotes tumor growth by increasing tumor angiogenesis [14]. Therefore, we also examined the effect of exogenous HA on tumor growth. As shown in **Fig. 4**, no significant
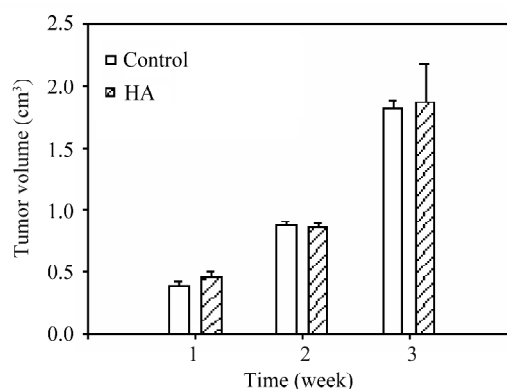
(A)

control   HA

(B)
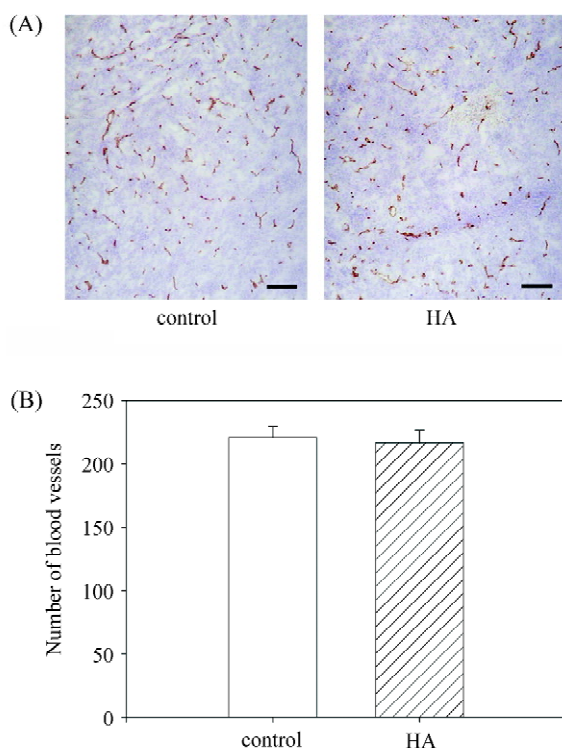
Fig. 3   Effect of exogenous hyaluronan (HA) on tumor angiogenesis
(A) Frozen sections of implanted tumors were stained for endothelial cell marker PECAM-1. Bar=100 μm. (B) The positive vessels of the highest density in a section were counted and normalized to the number.

Fig. 4   Effect of exogenous hyaluronan (HA) on tumor growth
Tumor length and width were measured weekly, and the tumor volume was calculated as length×width^2×0.52.

difference between the control tumors and HA-treated tumors was found. This result is consistent with the fact that exogenous HA has no significant effect on tumor angiogenesis.

Effect of exogenous HA on lymph node metastasis of Hca-F cells

It was suggested that tumor lymphangiogenesis might contribute to tumor lymphatic metastasis [2,3], so we further examined whether exogenous HA has any effect on lymphatic metastasis concomitantly with promoting lymphangiogenesis. Previously, Hca-F cells have been demonstrated to develop lymphatic metastasis selectively

[19]. In this study, superficial axillary, deep axillary and inguinal lymph nodes (on right side) were examined microscopically. The percentage of lymph node metastasis, calculated as the number of positive lymph nodes per total number of lymph nodes examined, was investigated. As shown in **Table 1**, the percentage of lymph node metastasis for control tumors is 53.3%, and 46.7% for HA-treated tumors. It was therefore concluded that exogenous HA does not significantly affect lymph node metastasis of Hca-F cells.

Discussion

Recent experimental studies have demonstrated the importance of lymphangiogenic growth factors VEGF-C and VEGF-D in tumor lymphangiogenesis and lymph node metastasis. In this study, we found that exogenous HA can induce tumor lymphangiogenesis and promote intralymphatic tumor growth. These results indicate that HA, in addition to VEGF-C and VEGF-D, may also play an important role in regulating tumor lymphangiogenesis.

Our results show that exogenous HA induces tumor lymphangiogenesis within xenografts. The HA-induced lymphatic vessels are hyperplastic, because the number

Table 1   Effect of exogenous hyaluronan (HA) on lymphatic metastasis of Hca-F cells

Group	Tested mice	Lymph node number	Metastatic mice	Metastatic lymph node number	Lymph node metastasis (%)
Control	5	15	5	8	53.3
HA	5	15	5	7	46.7

of lymphatic vessels is remarkably elevated and the frequent presence of LYVE-1⁺ cells indicates that new lymphatic vessels are continuously coming into being. Furthermore, our results demonstrate that HA selectively induces tumor lymphangiogenesis without affecting tumor angiogenesis. The differential effect of HA on tumor angiogenesis and lymphangiogenesis may be partly attributable to the different receptors for HA on blood vascular endothelium (BEC) and lymphatic vascular endothelium (LEC). CD44 and RHAMM are the receptors for HA on BEC, and both of them can mediate the signaling of proliferation and migration for BEC [20]. However, on LEC, the receptor for HA is LYVE-1, which is homologous to CD44 but largely restricted to LEC [21]. Although the function of LYVE-1 attracts much attention, it is not known whether LYVE-1 also participates in lymphangiogenesis. The identification of the true function of LYVE-1 will likely come from a whole animal study with an LYVE-1 knockout mouse, which is currently underway [22]. Additional mechanisms may also be involved in HA-induced lymphangiogenesis, owing to the unique properties of HA. For instance, the increase of HA concentration in local tissue might change the assembly of tumor stroma, which in turn facilitates tumor lymphangiogenesis. Consistent with this hypothesis, Skobe *et al*. found considerable heterogeneity of lymphatic vessel density within VEGF-C-overexpressed tumors, the authors speculate that this may reflect variations in tumor microenvironment: a promising microenvironment is likely to be critical for intratumoral lymphangiogenesis [6].

VEGF family members have been implicated in angiogenic and lymphangiogenic signaling. VEGF-A and VEGF-B are primarily involved in angiogenesis, whereas VEGF-C and VEGF-D play predominant roles in the regulation of lymphangiogenesis [23]. In additional experiments, we also explored the potential role of HA in regulating the expression of lymphangiogenic factors. The results showed that HA is able to increase the expression of VEGF-D at both mRNA and protein levels in Hca-F cells; on the other hand, HA has no significant effect on the expression of VEGF-A and VEGF-B (data not shown). These results suggested a potential role of VEGF-D in mediating HA-induced lymphangiogenesis, and also explain, at least partly, why HA can promote lymphangiogenesis without affecting angiogenesis. Other mechanisms may also contribute to this effect. It has been demonstrated that the promotion of angiogenesis by HA is dependent on its molecular size [12]. It was shown that HA oligosaccharides, but not the high molecular size of HA, induces endothelium proliferation and migration *in vitro* and angiogenesis *in vivo*. A

related finding is that tumor cells often exhibit elevated levels of not only HA itself but also hyaluronidase, which renders tumor cells the promoted ability to internalize and degrade HA [24]. Because little effect of HA on tumor angiogenesis was detected in this study, we postulated that HA might act in the form of high molecular size, instead of the form of HA oligosaccharides.

The data showed that HA-induced tumor lymphangiogenesis is accompanied with vast intralymphatic tumor growth. This phenomenon is very similar to the observation in another experimental model with overexpressing lymphangiogenic growth factor VEGF-C [9]. Tumor lymphangiogenesis has been taken as the immediate cause for the intralymphatic tumor growth, because lymphangiogenesis facilitates the interaction of tumor cells and lymphatic vessels [9]. Thus, it is likely that HA promotes tumor cells to infiltrate into lymphatic vessels indirectly through tumor lymphangiogenesis. Of course, we currently cannot exclude the possibility that HA may alternatively promote the intravasation of tumor cells directly by facilitating the interaction between tumor cells and LEC: HA sequestered by LYVE-1 at the lumina surface and intercellular junctions might provide en route a pathway for intravasation of CD44⁺ tumor cells into the lymphatic vessels. Consistent with this, a recent clinical report indicates that LYVE-1/HA may play a role in tumor lymphatic invasion [25].

HA-promoted lymphangiogenesis was shown to have no association with increased metastasis. Although lymphangiogenesis has been proposed to contribute to metastasis, their relationship remains confused. Lymphangiogenesis can be dissected from subsequent metastasis [25]. It was also shown that, in the absence of HA and lymphangiogenesis, tumor cells can still display lymph node metastasis, suggesting lymphangiogenesis is not necessary for metastasis. Considering the fact that HA fails to increase the metastasis rate, we postulated that lymphangiogenesis might play a minor role in the commitment of metastasis. Notably, we do detect the intralymphatic growth of tumor cells in the presence of HA, thus it can not be precluded that lymphangiogenesis may contribute to metastasis extent instead of metastasis rate.

It is believed that the interaction between tumor cells and the surrounding microenvironment plays a key role in tumor progression. Being the basic constituents of this microenvironment, tumor stroma should be taken as a functional pool for bioactive molecules rather than only a structural unit [26]. In the present study, we provide first-hand evidence that HA, which is usually elevated in malignant tumor stroma and multifunctional for malignancy,

may also play an important role in tumor lymphangiogenesis and promote intralymphatic tumor growth. To our knowledge, our data provide the first insight into the correlation between HA and tumor lymphangiogenesis. However, it is necessary to further explore the underlying mechanisms, especially to determine whether the interaction between HA and LYVE-1 is directly involved in tumor lymphangiogenesis and the intravasation of tumor cells into lymphatic vessels.

Acknowledgements

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