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Blockade of Hyaluronan Inhibits IL-2-Induced Vascular Leak Syndrome and Maintains Effectiveness of IL-2 Treatment for Metastatic Melanoma

Hongbing Guan, Prakash S. Nagarkatti, and Mitzi Nagarkatti

Vascular leak syndrome (VLS) is a life-threatening toxicity induced during IL-2 treatment of cancer patients. The mechanism of IL-2-induced VLS is still poorly understood. At present, there is no specific therapy for VLS. Previous studies from our laboratory demonstrated that hyaluronan (HA), a large glycosaminoglycan, abundant in the extracellular matrix and on the cell surface, caused a marked increase of IL-2-induced VLS in the lungs and liver of C57BL/6 mice. Conversely, blockade or knockout of its major receptor, CD44, resulted in a marked decrease of VLS, thereby suggesting a role for HA in VLS. In this study, we report a novel means to prevent IL-2-induced VLS by blocking endogenous HA with HA-specific binding peptide, Pep-1, a newly isolated peptide which specifically binds to soluble, cell-associated, and immobilized forms of HA. Our results demonstrated that blocking HA with Pep-1 dramatically inhibited IL-2-induced VLS in both normal mice as well as in mice bearing melanoma. Moreover, Pep-1 treatment maintained the effectiveness of IL-2 and prevented the metastasis of melanoma. IL-2-induced emigration of lymphocytes across the endothelium and cytotoxicity against tumor by lymphokine-activated killer cells were not affected by Pep-1. Instead, use of Pep-1 maintained endothelial integrity and reduced their apoptosis during IL-2-induced VLS. These data suggested that HA plays a critical role in regulating endothelial cell damage and induction of IL-2-mediated VLS. Also, blockade of HA using Pep-1 could constitute a novel therapeutic modality to prevent IL-2-mediated toxicity, thereby facilitating the effectiveness of high-dose IL-2 in the treatment of metastatic melanomas. The Journal of Immunology, 2007, 179: 3715–3723.

The mechanism of IL-2-induced VLS is still poorly understood. Direct effects of IL-2 on endothelial cells (9, 10), or through resultant cytokines such as TNF-α, IL-1, and IFN-γ (2), have been previously described. Cellular mechanisms involving CD8+ T cells, NK, NK, and PMN possibly play a role (11–13). The precise pathogenic role played by the different factors in VLS induction still needs further elucidation. Some studies showed that inhibitors of NO synthase are able to improve IL-2-induced VLS (14–16). It is also becoming increasingly clear that vascular endothelial cell damage is the major histopathological feature in IL-2-induced VLS (17–19).

Previous studies from our laboratory suggested a possible role of hyaluronan (HA) in the regulation of VLS (19). Administration of HA caused a marked increase in IL-2-induced VLS in the lungs and liver of C57BL/6 mice. LAK cytotoxicity against endothelial cells was enhanced with HA administration. The HA-treated mice showed endothelial cell damage, increased perivascular lymphocytic infiltration, and increased edema. When its major receptor, CD44 molecule, was blocked with anti-CD44 Ab, the IL-2-induced VLS was dramatically inhibited (19). Furthermore, the IL-2-induced VLS was markedly reduced in CD44 knockout mice (20, 21). However, our experiments did not clearly establish the roles played by HA because CD44 is not the exclusive receptor for HA and we did not block the HA molecule with specific binding. Therefore, it remains to be determined whether HA is directly involved in the development of IL-2-induced VLS.

The current report specifically addresses the role of HA in the development of IL-2-induced VLS and its implication in melanoma immunotherapy. The studies were based on specific blockade of HA with a HA-specific binding peptide, Pep-1 (22). The outcome may unveil a new mechanism for the induction of VLS and a new strategy in the treatment of patients with vascular injury.
Materials and Methods

Mice and rIL-2

Female C57BL/6 (6–8 wk of age) mice were purchased from the National Institutes of Health. All animals were housed in the University of South Carolina Animal Facility (Columbia, SC). All animal procedures were performed according to the National Institutes of Health guidelines under protocols approved by the Institute of Animal Care and Use Committee of the University of South Carolina.

rIL-2 was provided by the National Cancer Institute Biological Resources Branch (Rockville, MD).

Synthesis of Pep-1 and control peptide

Pep-1 (GAHWQFNLTVR) and scrambled control peptide (SC) (WRH GFALTAVNQ), both with an amidated GGGS linker (22), were synthesized by GenScript. We administrated the peptides at a dose of 1 mg/mouse i.p.

Quantification of VLS

VLS was induced by injection of IL-2 as previously described (17, 19–21). Groups of four to five mice were injected i.p. with 75,000 U of rIL-2 or PBS as a control, three times a day for 3 consecutive days. On day 4, the mice received one injection and 2 h later were injected i.v. with 0.1 ml of 1% Evan’s blue in PBS. After 2 h, the mice were exsanguinated under anesthesia, and the hearts were perfused with heparin in PBS until lungs and livers were blanched. The lungs and livers were harvested and placed in formamide at 37°C overnight. The Evan’s blue in the organs was quantified by measuring the absorbance of the supernatants at 650 nm with a spectrophotometer. In experiments examining the effect of Pep-1 on IL-2-induced VLS, mice received 1 mg of Pep-1 or SC with the every first injection of IL-2. The VLS seen in IL-2-treated mice was expressed as percentage of increase in extravasation of Evan’s blue when compared with that of the PBS-treated controls and was calculated as: \[ \frac{OD \text{ of dye in the organs of IL-2-treated mice} - OD \text{ of dye in the organs of PBS-treated controls}}{OD \text{ of dye in the organs of PBS-treated controls}} \times 100. \] Each mouse was individually analyzed for vascular leak, and data from four to five mice were expressed as mean \(\pm\) SE percentage increase in VLS in IL-2-treated mice when compared with that seen in PBS-treated controls.

Histological analysis of cell infiltration

Groups of four mice were induced with VLS as described above. On day 4, the lungs and livers were fixed in 10% formalin solution. The organs were embedded in paraffin, sectioned, and stained with H&E as described elsewhere (17, 19–21). Perivascular infiltration was scaled by counting the number of lymphocytes infiltrating the vessel and averaging the minimum and maximum range for each group. Four samples per mouse were analyzed and a minimum of four mice were included.

Electron microscopic studies of vascular injury

Tissue samples were fixed in 5% glutaldehyde/4.4% formaldehyde/2.75% picric acid in 0.05 M sodium cacodylate buffer (pH 7.4), washed in a...
sodium cacodylate buffer, postfixed in osmium tetroxide, embedded in Polybed 812 resin (Polysciences), and studied with an electron microscope as described previously (17, 20).

**TUNEL staining**

Cell apoptosis was evaluated with the conventional TUNEL assay using a DeadEnd Colorimetric TUNEL System from Promega. The assay was conducted according to the manufacturer’s instructions. Numbers of TUNEL-positive stained endothelial cells were counted in five vessels of each slide and the data are expressed as mean ± SE of apoptotic cells per vessel.

**Immunohistochemical staining of HA**

To characterize HA expression in lungs, tissues were fixed in 4% paraformaldehyde. Paraffin-embedded tissue sections were made for the staining. Sections were treated with 10 mM sodium citrate buffer (pH 6.0) to unmask Ags and with 1% BSA to block nonspecific binding. Endogenous peroxidase was blocked with 1% H2O2. Endogenous biotin and streptavidin binding were blocked using a Streptavidin/Biotin Blocking Kit (Vector Laboratories) per the manufacturer’s instructions. Sections were incubated with biotinylated hyaluronan-binding protein (HABP) (1 μg/ml; US Biological) in PBS containing 1% BSA overnight at 4°C, streptavidin-HRP (Biocare Medical) for 30 min at room temperature, and developed by incubating with diaminobenzidine (Biocare Medical). Cell nuclei were revealed with hematoxylin. The stained cells were evaluated under a microscope.

**Generation of LAK cells and 51Cr release assay**

LAK cells were prepared as previously described (21, 23). For generation of LAK cells in vitro, mouse splenocytes were prepared, adjusted to 5 × 10^6/ml, and cultured in vitro for 48 h with 1000 U/ml IL-2 in RPMI 1640 containing 10% FBS. The cells were harvested, and viable cells were purified by density gradient centrifugation using NycoPrep (Cedarlane Laboratories). Such cells will be referred to as LAK cells. In experiments examining the effects of HA on LAK cell development in vitro, Pep-1 (100 μg/ml) was added to the cultures at the beginning of the 48-h culture. For generation of LAK cells in vivo, mice were treated with IL-2 as described above. On day 4, the spleens were harvested and prepared into a single-cell suspension using a laboratory homogenizer (Stomacher; Tekmar). Removal of erythrocytes and separation of viable cells were done by density centrifugation using NycoPrep.

LAK cells generated in vitro and in vivo were tested for cytotoxicity against P815 tumor, YAC-1 tumor, B16F10 tumor, or TME endothelial cells using the 51Cr release assay (23). Briefly, 1 × 10^5 target cells were labeled with 100 μCi of 51NaCrO4 (MP Biomedicals) at 37°C for 1 h, washed sections were treated with 10 nM sodium citrate buffer (pH 6.0) to unmask Ags and with 1% BSA to block nonspecific binding. Endogenous peroxidase was blocked with 1% H2O2. Endogenous biotin and streptavidin binding were blocked using a Streptavidin/Biotin Blocking Kit (Vector Laboratories) per the manufacturer’s instructions. Sections were incubated with biotinylated hyaluronan-binding protein (HABP) (1 μg/ml; US Biological) in PBS containing 1% BSA overnight at 4°C, streptavidin-HRP (Biocare Medical) for 30 min at room temperature, and developed by incubating with diaminobenzidine (Biocare Medical). Cell nuclei were revealed with hematoxylin. The stained cells were evaluated under a microscope.

**FIGURE 3.** Histological studies on lungs and livers in IL-2-treated mice following administration of Pep-1. Mice received IL-2 or IL-2 + Pep-1 or IL-2 + SC as described in Fig. 1. Lungs and livers from the treated mice were harvested after the last IL-2 injection and preserved in 10% formalin solution. Sections were stained with H&E. Arrows, Perivascular infiltration, consisting mostly of lymphocytes. The level of perivascular infiltration was determined by counting the number of cells infiltrating a venule. The data depict the mean ± SE of sections from four individual mice. A, Lung histopathology; B, Liver histopathology; C, Level of perivascular infiltration in lungs; and D, Level of perivascular infiltration in livers. Original magnification, ×200.

**FIGURE 4.** Ultrastructural studies on lungs in IL-2-treated mice following administration of Pep-1. Mice received PBS, IL-2, or IL-2 + Pep-1 or IL-2 + SC as described in Fig. 1. IL-2-treated mice with significant damage to the endothelial cells. Cellular debris (CD) from former endothelial cells is found in the blood vessel lumen. Some of the endothelial cell morphology has been lost, with only extended cell membrane remnants remaining. Three RBC fill the lumen. 3, IL-2 + Pep-1-treated mice with normal endothelial cell morphology. The cell membranes and cytoplasmic contents are well defined and closely adhere to the basal lamina. The intact nucleus is closely opposed to the intact basal lamina. Two RBC fill the lumen. 4, IL-2 + SC-treated mice with the endothelial cell damage similar to 2. Original magnification, ×20,000.
three times in RPMI 1640 culture medium, and adjusted to 1 × 10^5 cells/ml. In brief, 1 × 10^4 target cells were plated into 96-well U-bottom plates with varying numbers of LAK cells depending on the respective E:T cell ratios. In these experiments, the LAK cells were defined as the effector cells that mediate lysis of the ^51^NaCrO_4-labeled endothelial cells or tumor cells, which were defined as the target cells. In some groups, target cells were incubated with Pep-1 (100 μg/ml) for 2 h at 37°C before they were added into the plates. Spontaneous release was determined by culturing the target cells alone, and the maximum release was determined by incubating the target cells with 1% SDS. The supernatants were harvested after a 4-h culture, and the radioactivity was measured using a Microbeta counter (PerkinElmer). Percentage of killing efficiency of LAK cells was calculated as percent cytotoxicity = [(sample cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm)] × 100. The data are represented as mean ± SE. Each group had four wells.

Flow cytometry studies of HA expression

To examine surface HA expression, cells were incubated for 30 min at 4°C with biotinylated HABP (0.25 μg/million cells) and then labeled with PE-conjugated streptavidin (BD Pharmingen). The staining was evaluated with a flow cytometer (CXP 500; Beckman Coulter).

To examine HA expression on TME cells, LAK cells were added to the culture of TME cells at a cell ratio of 3:1 in RPMI 1640 medium for certain time periods in the absence of IL-2. The coculture was washed extensively with the culture medium to remove LAK cells; the adherent TME cells were collected by trypsin treatment and stained for HA examination as above as well as stained with FITC-CD3 (BD Pharmingen).

Induction of VLS in tumor model

To create a melanoma model, C57BL/6 mice were implanted with B16F10 cells through i.v. injection. Several days later, mice received IL-2 for induction of VLS and/or Pep-1 as described before. We either implanted 7 × 10^7 B16F10 cells along with induction of VLS at day 7 after implantation, or 1 × 10^7 B16F10 cells along with induction of VLS at day 5 after implantation (we got similar results with both protocols). To evaluate tumor metastasis, black nodules were counted under a microdissecting microscope on the surface of lung specimens. These nodules exhibited characteristic histological features of metastatic melanomas (data not shown). The quantification of VLS was performed as described before.

Statistical analysis

The differences between experimental groups were analyzed using the Student t test with p < 0.05 being considered statistically significant.

Results

Pep-1 inhibits VLS in C57BL/6 mice

We first examined whether blocking HA could affect IL-2-induced VLS in C57BL/6 mice. Previous studies from our laboratory demonstrated that administration of HA caused a marked increase of IL-2-induced VLS in C57BL/6 mice; in contrast, CD44 knockout mice or mice treated with anti-CD44 mAb revealed markedly decreased VLS (19–21). Thus, we speculated that IL-2-induced VLS could be inhibited by blocking HA-CD44 interaction. To examine this, we administrated Pep-1 in mice. Pep-1 is a newly isolated peptide that specifically binds to soluble, cell-associated, and immobilized forms of HA. It has been confirmed that Pep-1 inhibits HA function by blocking its molecular interaction with HA-binding proteins (22, 24). Figure 1 shows a representative experiment that displayed VLS induced following IL-2 administration in the lungs and liver when compared with the PBS-treated group. However, mice that received IL-2 and Pep-1 showed a statistically significant decrease in VLS. The control mice that received IL-2 plus SC did not show any difference when compared with the IL-2-treated group. These experiments were repeated three times with consistent results. These results suggested that blockade of HA using Pep-1 can prevent IL-2-induced VLS.

Pep-1 inhibits VLS in melanoma-bearing mice with maintenance of IL-2 effectiveness

Although IL-2 therapy is effective in treating metastatic melanoma, it is not widely used due to severe toxicity, mainly VLS. The above results prompted us to further investigate whether we
can block VLS using Pep-1 in melanoma-bearing mice treated with IL-2. The B16F10 melanoma cell line does not express HA in vitro culture (25), but it does in vivo probably because of stimulation from the specific tumor microenvironment consisting of lactate and thus acquires an aggressive phenotype (26). To this end, we injected B16F10 melanoma cells into C57BL/6 mice through i.v. injection. On day 7, these mice received IL-2 and/or Pep-1 to induce VLS as described above. After the last injection (day 11 of tumor transplantation), we studied VLS in lungs and liver as well as tumor metastasis (as black nodules) in the lungs. These nodules exhibited characteristic histological features of metastatic melanoma (data not shown).

Our results demonstrated a significant decrease of VLS in the lungs and liver in the Pep-1-treated group which was similar to the results from nonmelanoma mice (Fig. 2). Moreover, mice receiving IL-2 treatment exhibited significantly \( p < 0.001 \) reduced numbers of lung metastatic lesions when compared with the non-IL-2-treated mice. Pep-1 or SC alone did not affect lung metastasis as seen from the same levels of metastatic lesions when compared with PBS-injected mice (data not shown). These results indicated that Pep-1 can attenuate IL-2-mediated toxicity in lungs while maintaining the effectiveness of IL-2 treatment on metastatic melanoma.

**Pep-1 protects endothelial cell integrity**

We have shown previously that IL-2-induced VLS results from actual damage to the endothelial cells potentially caused by IL-2-activated LAK cells (17–19). To demonstrate that a decrease in VLS in Pep-1-treated mice was related to endothelial cell damage, ultrastructural studies of the lungs were performed. As shown in Fig. 4, lungs from PBS-injected (control) mice displayed no morphological features of cell damage. In contrast, lungs from IL-2-injected mice revealed extensive damage to the basal lamina and endothelial cells. Cellular debris from endothelial cells were found in the blood capillary lumen. Some of the endothelial cells were

**Pep-1 does not affect lymphocytes migrating to interstitium**

Inasmuch as HA is involved in lymphocyte homing to organs (24), histopathological studies were conducted to investigate whether the decrease in VLS in Pep-1 treatment was due to the inability of lymphocytes to migrate to the lungs and liver. Mice were injected with PBS, IL-2, Pep-1, or SC as described in Fig. 1. On day 4, the organs were harvested and stained with H&E. The lymphocyte extravasation was examined under a microscope. The PBS-treated mice did not exhibit any perivascular infiltration in the lungs and liver (Fig. 3, A and B). In contrast, IL-2-treated mice showed significant perivascular lymphocytic infiltration both in lungs and liver. Notably, IL-2 plus Pep-1-treated mice did exhibit similar levels of perivascular infiltration as the IL-2-treated mice. The degree of infiltration was also measured by counting the number of lymphocytes infiltrating each vessel and averaging the range for each group (Fig. 3, C and D). These results showed that IL-2 or IL-2 plus Pep-1 treatment had similar levels of perivascular infiltration. These data suggested that the decrease in IL-2-induced VLS seen in IL-2 plus Pep-1-treated mice was not due to the inability of lymphocytes to migrate to the lungs and liver.

**FIGURE 6.** Increased HA expression in IL-2-treated mice. IL-2 was used to induce VLS as described in Fig. 1. In situ HA expression in vivo was examined by immunohistochemistry using biotin-conjugated HABP as the probe. Biotin-HABP was labeled with streptavidin-HRP and developed with diaminobenzidine. Cell nuclei were stained with hematoxylin. Positive cells show brown-stained cytoplasm and cell surface. Upper row, PBS-treated control mice; bottom row, IL-2-treated mice. Original magnification, \( \times 400 \).

**FIGURE 7.** Increased HA expression on endothelial cells and LAK cells. HA expression on cells was examined by flow cytometry. The gates were set on viable cells. A. The data from TME cells cultured alone or in the presence of LAK cells for 4, 6, or 11 h. The cells from cocultures were stained with FITC-CD3, biotinylated HABP, and PE-conjugated streptavidin. B. LAK cells were stained with PE-conjugated streptavidin (labeled SAV-PE) or with biotinylated HABP and PE-conjugated streptavidin (labeled LAK). As a control, spleen cells were stained with biotinylated HABP and PE-conjugated streptavidin (labeled splenocytes). C. B16F10 cells were stained with PE-conjugated streptavidin (labeled SAV-PE) or with biotinylated HABP and PE-conjugated streptavidin (labeled HABP).
severely damaged, with only extended cell membrane remnants remaining. In contrast, Pep-1 plus IL-2-treated mice exhibited morphologically almost normal endothelial cells pressed against the basal lamina while only a few endothelial cells showed minor damage. Again, SC did not show any protection of VLS, corroborated by extensive damage similar to IL-2-treated mice. The ultrastructural results suggested that Pep-1 can protect endothelial cell damage from IL-2 toxicity.

**Pep-1 protects from IL-2-mediated endothelial cell apoptosis**

In addition to the ultrastructural damage to the endothelial cells, we examined whether endothelial cells underwent apoptosis following IL-2 treatment. So far, there have been no reports on involvement of apoptosis in IL-2-induced VLS. It is known that inhibitors of apoptosis are protective against acute lung injury (27–29). We speculated that apoptosis could be a mechanism for endothelial cell damage. Hence, we applied TUNEL staining to examine apoptosis in lung sections.

Our results showed that IL-2-treated mice exhibited a large number of endothelial cells that had undergone apoptosis, as determined by TUNEL-positive staining. In contrast, Pep-1 plus IL-2-treated mice showed a minimal number of apoptotic cells (Fig. 5). These data suggested that Pep-1 administration may prevent IL-2-induced VLS by blocking apoptosis in endothelial cells.

**HA expression is enhanced in IL-2 treatment**

Inasmuch as Pep-1 specifically binds to HA, we next investigated HA expression in endothelial cells and tested whether following IL-2 administration, its regulation is altered. To this end, we stained HA in lung sections using the HA-specific probe HABP. We found that HABP stained not only endothelial cells but also epithelium of airways. In the IL-2-treated group, intensity of the staining was dramatically increased (Fig. 6).

Next, we tested whether during LAK cell interaction with endothelial cells there would be altered expression of HA on the latter cells. To this end, we cultured endothelial cells (TME, a murine endothelial cell line) with LAK cells and assessed staining of HA on endothelial cells using flow cytometry. These cells were cultured at a ratio of 1 TME to 3 LAK cells for 4–20 h. The majority of TME cells died after 16 h. We observed an increased expression of HA on TME cells, and the expression increased with the culture duration studied (Fig. 7). IL-2 alone failed to induce such
an up-regulation (data not shown), thereby suggesting that cell-cell contact between IL-2-induced LAK cells and endothelial cells may be critical for increased expression of HA on endothelial cells. We also observed an increased expression of HA on a significant proportion of spleen cells cultured with IL-2 which may represent LAK cells when compared with nonactivated splenocytes; however, the extent of expression was much lower than that on TME cells (Fig. 7).

Pep-1 does not affect development and cytotoxicity of LAK cells

Direct killing of endothelial cells by LAK cells represents one possible mechanism in IL-2-induced VLS (19, 21, 30, 31). Therefore, we investigated whether Pep-1 affected LAK cytotoxicity. To this end, we generated LAK cells from IL-2 or IL-2 plus Pep-1-treated mice as described in Materials and Methods, and examined their cytotoxicity against TME, YAC-1, P815, and B16F10 target cells using the standard chromium release assay. As shown in Fig. 8, there was no difference in cytotoxicity of LAK cells between IL-2 treatment and IL-2 plus Pep-1 treatment for each target.

We also tested the effect of Pep-1 in vitro. To this end, we generated LAK cells in vitro and Pep-1 was added at the beginning of the culture. The results showed that Pep-1 did not affect the killing efficiency of LAK cells against any of the target cells tested (Fig. 9). Together, these data suggested that Pep-1 did not affect the development and cytotoxicity of LAK cells.

Blockade of HA on target cells can protect cells from LAK cell-mediated killing

The above results demonstrated that either in vivo or in vitro blockade of HA did not interfere with the development and function of LAK cells. However, our experiments also demonstrated that blockade of HA protected endothelium integrity and decreased endothelial cell apoptosis. To explore this phenomenon further, we studied the role of HA on target cells. We examined surface HA expression on NK-sensitive and -resistant cell lines, such as YAC-1 and P815 as well as on B16F10 melanoma cells used in this study, by flow cytometry using HABP as specific probe. We noted that only YAC-1 expressed significant levels of HA, while P815 failed to express HA (data not shown). Also, the B16F10 melanoma cells used in this study failed to express significant levels of HA (Fig. 7).

To study the role of HA on target cell killing, we preincubated YAC-1, P815, B16F10, and TME target cells, with Pep-1 for 2 h at 37°C before addition of effector LAK cells. The results showed that LAK cell-mediated killing of Pep-1-treated HA-bearing YAC-1 and TME targets was significantly decreased when compared with the respective controls. However, pretreatment with Pep-1 failed to alter the lysis of HA-deficient P815 and B16F10 tumor targets (Fig. 10). These data suggested that blockade of HA on endothelial cells by Pep-1 protects them from LAK cell-mediated killing and this may explain why Pep-1 administration in vivo decreases IL-2-induced VLS.

Discussion

Antimalignancy or antiretroviral infection therapy with IL-2 administration has been hindered by the severe toxicity of IL-2 leading to VLS. So far, there are no effective measures to overcome its toxicity. The present study suggested that Pep-1 could be effective to treat vascular leak and that HA could be involved in the induction of this syndrome.

HA is a large, unbranched, nonsulfated glycosaminoglycan composed of alternating N-acetylgalactosamine and glucuronic acid subunits. The views about its function are rapidly expanding because of the latest explorations. Its functions were summarized as filler theory, adhesion theory, and signaling theory, in which, far from an inert structural biopolymer, HA represents a multifunctional carbohydrate mediator of immune processes (24). Our findings propose that HA could be involved in the pathophysiological process of the endothelial cell damage not only in IL-2-induced VLS but in other endothelial damage-related diseases. We propose that interfering function or signal transduction of HA constitutes a novel approach to prevent VLS. The current study also sheds new light in understanding the mechanism of IL-2-induced VLS.

HA expression is a tightly regulated process by exogenous stimuli. Cytokines and chemokines such as IL-1β, TNF-α, TGFβ, and IL-15 can up-regulate HA expression on endothelial cells (32–34). In the current study, we noted that HA expression on endothelial cells was significantly elevated following IL-2 treatment. It should be noted that this may not result from the direct effect of IL-2 on endothelial cells but may result from a large number of other chemokines and cytokines that are triggered during VLS. For example, in an earlier study, we showed that during IL-2-mediated VLS, the serum level was dramatically increased for IL-6, IL-10, IL-12(p40), and RANTES. Also, the splenocytes that developed into LAK cells in vitro with IL-2 produced high levels of IL-6, IL-12(p40), IFN-γ, and RANTES (23). Such cytokines and chemokines may play a key role in inducing the expression of HA on the endothelial cells in vivo. Additionally, our studies demonstrated...
that cell–cell contact between LAK cells and endothelial cells may be necessary to induce HA on the latter cells. Also, lactate from the tumor microenvironment has been shown to induce HA expression in B16F10 melanoma cells (26). In tumor-bearing mice, such a mechanism may also stimulate endothelial cells to synthesize HA.

Previous studies from our laboratory have shown that IL-2–activated LAK cells express increased levels of CD44 and that signaling through CD44 can trigger cytolytic function, leading to killing of target cells that express HA such as the endothelial cells (17, 19–21, 31, 35). In the current study, we noted that treatment with Pep-1 caused a decrease in apoptosis and endothelial damage triggered by IL-2. This raises a question of what mechanisms account for the increased resistance of endothelial cells to the damage? We would like to propose several possibilities. First, we propose that blockade of HA could interrupt the recognition and contact between LAK cells and endothelial cells. HA has been shown to bind several different molecules, including CD44 (a primary receptor of HA), the receptor for HA-mediated motility (a second receptor of HA), link protein, aggreccan, versican, hyaluronectin, neurocan, liver sinusoidal endothelial HA receptor, inter-a-trypsin inhibitor-related proteins, brain-enriched HA binding, CD38, lymphtic vessel endothelial HA receptor 1, and white fat/bone marrow/osteoblast HA-binding proteins (22, 36). Among them, HA-CD44 interaction has been considered as the primary reaction that plays a significant role in several immune functions, including cytotoxicity. For example, inflammatory signals increased HA expression on endothelial cells and CD44 expression on activated LAK cells as well as led to enhancement of the adhesion between LAK cells and endothelium (16, 34). Second, we propose that blockade of HA could suppress the damage of NO on endothelial cells. HA fragments markedly stimulate inducible NO synthase mRNA and NO production in endothelial and Kupffer cells; IFN-γ could augment this stimulation (37). NO synthase inhibitors are able to prevent endothelial damage in IL-2–induced VLS (14–16). We speculate that blockade of HA could suppress inducible NO synthase mRNA and NO production, thereby decreasing the damage to endothelial cells, which is supported by the fact that IFN-γ level is increased in the serum of IL-2–induced VLS in mice (23). We assume a down-regulated IFN-γ production in Pep-1–treated mice, which needs further investigation. Third, we propose that the interaction between Pep-1 and HA could generate signals via TLRs to prevent apoptosis in endothelial cells, which is supported by the studies from other groups in which it was demonstrated that HA binds to TLR4 and TLR5 on endothelial cells (29, 38). Also, administration of Pep-1 can inhibit apoptosis of lung epithelial cells (29). Fourth, we propose that activated endothelial cells pass suppressive signals to inhibit LAK killing. Endothelial cells are not just a passive target; IL-2 and other mediators may induce an activated state in endothelial cells which expresses several kinds of molecules, such as the adhesion molecules ET-1 and cELAM-1 (39). Cytokines from endothelial cells, such as IL-2 and IL-15, can predispose NK cells to undergo apoptosis (40). When treated with Pep-1, the cytokine profile of the endothelial cells may experience dramatic change so as to deliver suppressive signals to the killer cells. Lastly, the endothelial-specific marker CD146 is related to the cell damage (41). It is possible that blockade of HA may affect CD146 expression and consequently the cell damage. Further studies are necessary to address these hypotheses.

The above explanations raise another question. Why is the ability of LAK cells to inhibit melanoma metastasis in vivo not inhibited? Our results showed that LAK cell-mediated killing of B16F10 tumor in vitro was not affected by blockade of HA. Moreover, in vitro-cultured B16F10 melanoma cells did not express HA (Ref. 25 and Fig. 9). Although B16F10 cells may express HA in vivo probably because of stimulation from the specific tumor microenvironment (e.g., lactate) (26), Pep-1 administration may not affect the tumor growth. For example, previous studies have shown that Pep-1 administration failed to inhibit local growth of s.c. inoculated tumor cells by repeated intratumor injections (26). Also, the in vitro proliferation of the tumor was not affected by adding Pep-1 or HA, thereby suggesting that targeting HA may not alter the growth and metastasis of B16F10 melanoma cells (26). We believe that the ability of Pep-1 to block HA-CD44 interaction does not interfere with the recognition, contact, and killing of B16F10 melanoma cells by LAK cells as shown in the current study. It is also possible that LAK cells use unique CD44 isoforms to interact with and kill endothelial cells vs tumor cells, as shown in our previous studies (21).

It should be noted that although IL-2 can activate LAK cells to mediate tumor killing, it can also expand CD4+CD25+ regulatory T cells in vivo and in vitro (42–44). Our previous study has observed an increased number of CD4+CD25+ regulatory T cells with IL-2 administration (23). Interestingly, we noted that the regulatory T cells also played a critical role in preventing IL-2–induced VLS resulting from endothelial cell injury inasmuch as depletion of T regulatory cells led to a significant increase in IL-2–induced VLS (23). Whether blockade of HA affects the function of T regulatory cells is worthy of further exploration. A variety of immunotherapeutic approaches have been tried in patients with metastatic melanoma with the best results being observed with IL-2 (45). This has led to the approval by the Food and Drug Administration to use a high-dose IL-2 regimen in this patient population. However, due to the severe toxicity associated with high-dose IL-2 therapy, efforts to develop a more tolerable form are being investigated. These include prolonged administration of lower doses or the combination of IL-2 with other cytokines, mAbs, or vaccines. Such research has not yet produced results superior to those seen with high-dose IL-2 alone (45). Our studies using Pep-1 to block CD44-HA interactions provide a novel approach to neutralize IL-2–induced VLS, which constitutes a major form of toxicity, while retaining the antitumor efficacy.

Disclosures

The authors have no financial conflict of interest.

References
