Inhibition of Tumor Growth In Vivo with a Soluble CD44-immunoglobulin Fusion Protein

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Summary

CD44H is the principal cell surface receptor for hyaluronate, which is a major glycosaminoglycan of the extracellular matrix. Expression of CD44H is enhanced in a variety of malignant tumors and correlates with tumor aggressiveness, supporting the notion that interaction between CD44H and hyaluronate may play an important role in tumor growth and dissemination. In this report we show that in vivo tumor formation by human lymphoma Namalwa cells, stably transfected with CD44H, can be suppressed by a soluble human CD44H-immunoglobulin fusion protein. Disruption of the interaction between CD44H and its physiologic ligands may provide a novel strategy for controlling tumor growth in vivo.

Materials and Methods

Preparation of Soluble CD44Rg. CD44Rg and CD8Rg (used as a control) were prepared as previously described (6). Briefly, synthetic oligonucleotide-primed amplification of cDNA sequences encoding the extracellular domain of CD44H or CD8 was per-
formed in PCR. Oligonucleotides were designed to contain endonuclease restriction sites to facilitate subsequent insertion of amplified sequences into Ig expression vectors (6). Amplified sequences were subjected to appropriate restriction enzyme digestion and ligated to Ig vectors previously subjected to corresponding endonuclease cleavage. CD44Rg and CD8Rg vectors were introduced into COS cells by the DEAE-Dextran method (6), and 5-7 d posttransfection, supernatants were harvested and Rg molecules purified on Protein A trisacryl beads (Pierce Chemical Co., Rockford, IL). CD44Rg and CD8Rg fusion proteins were eluted with 0.1 M acetic acid pH 4.5, dialyzed overnight, and purified protein concentration determined in ELISA assays. Typically 1-3 μg of Rg/ml of supernatant were obtained.

**Cell Lines.** Development of stable CD44 transfectants in the human Burkitt lymphoma cell line Namalwa was described previously (8, 16). CD44 N.2 transfectants, selected for high expression of CD44H (16), were maintained in DMEM, (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Laboratories), gentamycin, and 500 μg/ml hygromycin (Sigma Chemical Co., St. Louis, MO). Cells were periodically tested for conservation of CD44 expression by indirect immunofluorescence using anti-CD44 mAb F-10-44-2 (Accurate Chemical & Scientific Corp., Westbury, NY), and affinity-purified goat anti–mouse antibody (Cappel Laboratories, Malvern, PA). Antibody reactivity was analyzed on a fluorescence activated analyzer (Becton Dickinson & Co., Mountain View, CA).

**Determination of the Effect of CD44Rg Reactivity with Transfectants and the Effect of CD44Rg on Transfectant and Parental Cell Growth In Vitro.** CD44 + N2.1 tumor cells were cultured per well in RPMI medium (Gibco Laboratories) supplemented with 10% FCS, gentamycin, and 1% l-glutamine in 96-well, flat-bottomed microtiter plates (Falcon, Lincoln Park, N.J.) for 24 h. Purified CD44Rg or CD8Rg were added to the wells at the beginning of the culture period at concentrations of 12, 25, and 50 μg/ml. After the 24-h culture period, cells were pulsed with 1 μCi of [3H]thymidine for 4 h. Cells were then washed, harvested with an automatic PH.D. harvester (Cambridge Bioscience, Cambridge, MA), and [3H] incorporation determined. All cultures were done in triplicate. In addition, transfectants and parental cell lines were tested for possible binding of CD44Rg by indirect immunofluorescence. Cells were incubated with CD44Rg at concentrations ranging from 2-20 μg/ml on ice for 1 h, washed in PBS, incubated with an affinity-purified goat anti–human, fluorescein-labeled antibody for 30 min on ice, washed, fixed in 4% formaldehyde, and examined for CD44Rg binding on a FACScan® (Becton Dickinson & Co.).

**Determination of In Vivo Half-life of CD44 and CD8Rg.** Two groups of 6-8-wk old female BALB/c (nu/nu) mice (6/group) were injected with 500 μg of purified CD44Rg or CD8Rg intravenously. Serum samples were obtained from individual mice at various times after injection. At every time point, mice from which serum had been obtained were killed for immunohistological tissue examination. The concentration of CD44Rg and CD8Rg was determined in an ELISA specific for CD44 and CD8Rg, respectively. Briefly, 96-well ELISA plates were coated with 0.5 mg/ml of a monoclonal anti-CD44 antibody (A3) or a monoclonal anti-CD8 (OKT8) antibody. Serum samples from individual mice were diluted (1:10) with PBS and added to the plates. The second step was performed with an affinity-purified alkaline phosphatase-conjugated goat anti–human, IgG-specific antibody (Southern Biotechnology Associates, Birmingham, AL). This assay was found to detect between 1 and 10 ng of CD44Rg or CD8Rg.

**Introduction of Tumor Cells and Rg Molecules into Animals and Monitoring of Tumor Growth.** CD44 + N.2.1 tumor cells, resuspended at a concentration of 1.7 × 10⁷/ml either in PBS containing 300 μg/ml of CD44Rg or in PBS alone were injected intravenously into two groups of BALB/c (nu/nu) mice. Each mouse received 5.1 × 10⁶ N.2.1 tumor cells in 0.3 ml (five mice received cells in PBS and six mice received CD44Rg). Three subsequent daily injections of either PBS alone or CD44Rg (300 μg) in PBS were administered. Tumor growth was determined by measuring the concentration of human IgM in the serum of nude mice with a human IgM-specific ELISA as described previously (16), and confirmed by histological examination of organs at autopsy.

In a second set of experiments, CD44 + N.2.1 tumor cells were resuspended at 1.7 × 10⁷/ml in PBS containing either 500 μg/ml of CD44Rg, or 500 μg/ml of CD8Rg or in PBS alone, and were injected intravenously into three groups of BALB/c (nu/nu) mice. Each mouse received 5.1 × 10⁶ N.2.1 tumor cells in 0.3 ml, as above, and three more daily injections of PBS alone (four mice), PBS with CD44Rg or CD8Rg (500 μg, 5 mice) were administered. Tumor growth was determined as above.

Parental, CD44 + Namalwa cells were resuspended at 1.7 × 10⁷/ml in PBS containing 500 μg/ml of sCD44-Ig or in PBS alone, and were injected intravenously into two groups of BALB/c (nu/nu) mice. Each mouse received 5.1 × 10⁷ Namalwa cells in a 0.3 ml volume. Subsequent injections and tumor growth evaluation were performed as above.

**Results and Discussion**

**CD44Rg and CD8Rg Do Not Alter CD44 + or CD44 − Namalwa Cell Growth In Vitro.** CD44Rg was prepared by ligating cDNA sequences encoding the extracellular domain of human CD44H to genomic sequences encoding the C region of human IgG, as described (6). Soluble human CD8Rg (6) was used as a control. To determine whether CD44Rg might interfere with growth of CD44 + tumor cells in vitro, CD44H − cells derived from a Namalwa CD44,5 tumor grown in subcutaneous tissues of a nude mouse (16) were selected for the highest level of CD44H expression by limiting dilution. A CD44H − subclone, N2.1, was isolated that expresses at least a 10-fold greater amount of surface CD44H than the parental cell line Namalwa-CD44,4,5, as determined by indirect immunofluorescent staining with a monoclonal anti-CD44 antibody (data not shown). N2.1 was cultured in vitro in the presence of various concentrations of CD44Rg or CD8Rg. After 24 h in culture, cells were pulsed with [3H]thymidine and harvested 4 h later. Neither CD8Rg nor CD44Rg altered the growth of CD44 + N2.1 or CD44 − Namalwa cells in vitro (data not shown), and neither reagent bound to either cell line as assessed by indirect immunofluorescence (data not shown). These observations indicate that CD44Rg does not react directly with N2.1 or parental Namalwa cells, and that its presence does not affect growth of either cell line in vitro. Any effect on tumor cell growth in vivo would therefore not be the result of CD44Rg interaction with the tumor cells, but rather from CD44Rg-mediated inhibition of tumor cell binding to substrate as a result of competition for specific binding sites.

**Determination of the In Vivo Half-life of Rg Molecules.** Before investigating whether CD44Rg can block tumor growth in vivo, we determined the half-life and distribution of CD44Rg in nude mice after intravenous administration. Two
groups of nude mice (6/group) were injected intravenously with 100 μg of CD44Rg or CD8Rg per mouse. Animals were bled at various time points after injection and serum levels of CD44Rg and CD8Rg determined using an ELISA specific for CD44Rg and CD8Rg, respectively. The ELISA assay allowed detection of as little as 1–10 ng of CD44Rg or CD8Rg in serum, respectively. The half-life of CD44Rg in nude mice ranged from 60 to 90 min (Fig. 1). In contrast, the half-life of CD8Rg was about 40 h. In an attempt to determine tissue penetration of the Rg molecules, we performed immunohistological staining for CD44Rg and CD8Rg in various tissues after in vivo injection. 2 h after injection, CD44Rg could be detected in organs that have a hyaluronate-rich ECM, the most intense staining being localized to the renal papilla (data not shown). In contrast, CD8Rg was not detected in any of these tissues by indirect immunofluorescence. The failure to detect CD8Rg by this approach does not exclude the possibility that soluble CD8 might penetrate tissues when injected intravenously, but suggests that CD8Rg does not recognize ligands in the tissues examined, which is consistent with earlier observations (6). These experiments provide evidence that intravenous injection of CD44Rg results in rapid removal of the fusion protein from the circulation, and suggest that the short serum half-life of CD44Rg is most likely due to its retention in tissues expressing specific ligands.

**Table 1. Tissue Distribution of Metastases derived from Animals Injected with N2.1 with PBS or CD44Rg or CD8Rg**

| Tissue       | PBS (8/9) | CD8Rg (4/5) | CD44Rg (3/11) |
|--------------|-----------|-------------|---------------|
| Bone marrow  | 8         | 4           | 3             |
| Spleen       | 0         | 0           | 0             |
| Kidney       | 2         | 0           | 0             |
| Peritoneum   | 4         | 1           | 0             |
| Gut          | 1         | 0           | 0             |

Combined results from both sets of experiments, at 300 and 500 μg of CD44Rg/injection are presented. The fraction of injected animals that developed tumor growth in each group is indicated in brackets. All three animals in the CD44Rg group which developed tumors had received the lower dose (300 μg) of CD44Rg/injection.

CD44Rg Inhibits Dissemination and Growth of CD44* Lymphoma Cells. We next attempted to determine whether administration of CD44Rg could block CD44H* N2.1 tumor cell growth in vivo. In the first set of experiments, CD44H* N2.1 tumor cells were resuspended at 1.7 × 10⁷/ml either in PBS containing 300 μg/ml of CD44Rg or in PBS alone, and were then injected intravenously into two separate groups of nude mice. Each mouse received 5 × 10⁶ N2.1 tumor cells in a 0.3 ml suspension. Initial injection was followed by one additional injection a day for 3 d of either PBS alone or PBS with 300 μg CD44Rg. Tumor growth was assessed by the quantitating Namalwa cell-derived human IgM in the serum of nude mice, based on the earlier observation that Namalwa cell-derived solid tumor growth was consistently associated with detectable levels of human IgM in murine serum (16). This allowed early detection and monitoring of tumor growth. However, in the event that certain tumors might lose the ability to secrete IgM, an autopsy was performed on all animals that died during the course of the experiments, and tumor presence confirmed by gross and histological examination (Table 1). All surviving animals were killed 100–130 d after initial tumor injection, and tissues examined for evidence of tumor growth. Tumor growth was significantly delayed and fewer animals developed tumors in the CD44Rg-treated group. 6 wk after tumor cell injection, 80% of the animals that had received N2.1 in PBS alone had died. In contrast, only one mouse receiving N2.1 and CD44Rg developed detectable tumor growth. 3 mo after tumor injection, two additional animals treated with CD44Rg developed tumors (Fig. 2 A).

In a second set of experiments, we increased the dosage of CD44Rg to 500 μg/injection and included an additional control group in which animals were injected with an identical number of tumor cells and 500 μg of CD8Rg (Fig. 2 B). All animals that had received CD8Rg, and 80% of the
animals that had received PBS died within 10 wk of tumor cell injection. However, none of the animals that had received CD44Rg revealed detectable tumor growth, even 100 d after the initial tumor cell injection (Table 1). These observations provide evidence that CD44Rg specifically inhibits N2.1 cell-derived tumor growth in nude mice.

However, similar experiments revealed that CD44Rg did not delay or block the development of tumors in animals injected with parental CD44− Namalwa cells (Fig. 2 C). The effects of CD44Rg are therefore most likely due to its ability to block interaction between CD44H on tumor cells and its ligands in host tissues.

CD44Rg appeared to be much more effective in retarding tumor growth than we had originally anticipated. In recent work, we reported that both parental Namalwa cells and Namalwa CD44H + transfectants produced tumors when injected either subcutaneously or intravenously into nude mice (16). However, CD44H + Namalwa transfectants produced tumors more rapidly and consistently than the parental cell line, while displaying a similar pattern of hematogenous dissemination. These observations were consistent with the notion that CD44H expression in lymphoma cells may promote the initiation of tumor growth, but may not be essential for tumor development. Based on these results, we had anticipated that administration of CD44Rg might reduce the in vivo growth rate of N2.1 tumors to the level of the parental cell-derived tumors. It is surprising, that CD44Rg was found to retard the growth of intravenously administered N2.1 tumor cells to a rate significantly lower than that of CD44− Namalwa tumors (Fig. 2, A and B). 10 wk after tumor injection, most of the animals that had received CD44− Namalwa cells, with or without CD44Rg, had developed tumors or died. No tumors, on the other hand, were observed in any of the animals injected with N2.1 cells and 500 µg of CD44Rg. Absence of tumor growth in these animals was confirmed at the macroscopic and microscopic level in all major organs. These observations raise the possibility that expression of CD44H may interfere with expression or functional ECM binding capacity of other, constitutive Namalwa cell surface receptors. High CD44H expressors, such as N2.1 cells, may therefore display an increased dependence on CD44H for interaction with the host tissue microenvironment and tumor formation.

Dissemination and metastatic growth of tumors are complex processes in which expression of several classes of adhesion molecules, which regulate tumor cell interaction with endothelium and ECM, play a major role (17, 20, 21). In the natural evolution of malignancies, only a fraction of cells that constitute the primary tumor are believed to display the phenotype necessary to develop metastatic growth (22, 23). Of that fraction, only a few cells are responsible for originating secondary growth. It is therefore reasonable to speculate that appropriate administration of soluble adhesion molecules, which block tumor cell interaction with endothelium or substrate, may contribute to limiting hematogenous tumor dissemination. The present experiments were designed to test the tumor dissemination and growth-blocking effectiveness of CD44Rg under conditions that strongly favor tumor development, by introducing large numbers of tumor cells into immunocompromised animals. To maximize the potential tumor-inhibiting action of CD44Rg, it was judged appropriate to inject the fusion protein and the tumor cells simultaneously. Although additional investigation will be necessary to determine whether soluble CD44 may have a clinical value as an auxiliary therapeutic reagent, the present observations suggest that CD44Rg may provide a useful tool for the study of tumor growth in vivo, as well as a potential means of controlling dissemination of CD44HS lymphoid malignancies.
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