Fragmented Hyaluronan Induces Transcriptional Up-regulation of the Multidrug Resistance-1 Gene in CD4+ T Cells*

Received for publication, February 2, 2006, and in revised form, October 10, 2006 Published, JBC Papers in Press, October 12, 2006, DOI 10.1074/jbc.M601030200

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P-glycoprotein, encoded by the multidrug resistance (MDR)-1 gene, expels various drugs from cells resulting in multidrug resistance. We found previously that interleukin-2, a lymphocyte-activation cytokine, induces P-glycoprotein expression on lymphocytes. Lymphocyte activation involves lymphocyte-activation cytokine, induces P-glycoprotein expression on lymphocytes by fragmented hyaluronan. Fragmented hyaluronan (especially the 6.9-kDa form), not native high molecular hyaluronan, induced translocation of YB-1, a specific transcription factor for YB-1, a specific transcription factor for the transcription of MDR-1 in lymphocytes by fragmented hyaluronan. Fragmented hyaluronan (especially the 6.9-kDa form), not native high molecular hyaluronan, induced translocation of YB-1, a specific transcription factor for MDR-1, from the cytoplasm into the nucleus and resulted in the transcription of MDR-1 and the expression of P-glycoprotein on lymphocytes in a dose-dependent manner. Transfection of YB-1 antisense oligonucleotides inhibited P-glycoprotein expression induced by fragmented hyaluronan. The fragmented hyaluronan induced significant P-glycoprotein expression on only activated CD4+ T cells, which highly expressed CD69, and resulted in excretion of intracellular dexamethasone added in vitro. Cyclosporin A, a competitive P-glycoprotein inhibitor, restored intracellular dexamethasone levels in CD4+ T cells. Anti-CD44 monoclonal antibody (Hermes-1) inhibited fragmented hyaluronan-induced YB-1 activation and P-glycoprotein expression in CD4+ T cells. We provide the first evidence that binding of fragmented hyaluronan to CD44 induces YB-1 activation followed by P-glycoprotein expression in accordance with activation of CD4+ T cells. Our findings imply that CD4+ T cell activation by fragmented hyaluronan, induced by characteristic extracellular matrix changes in inflammation, tumors, and other conditions, results in P-glycoprotein-mediated multidrug resistance.

The successful pharmacotherapy of various diseases, including tumors and autoimmune diseases, is often limited by multidrug resistance (1–3). Among the multiple mechanisms of multidrug resistance, overexpression of P-glycoprotein, a 170-kDa product of the multidrug resistance (MDR)-1 gene, has emerged as the major molecule involved in multidrug resistance during chemotherapy for various malignancies (1–6). Resistance to chemotherapy induced by P-glycoprotein is closely associated with the prognosis of human malignancies (1). P-glycoprotein is a member of the ATP-binding cassette transporter superfamily of genes and functions as an energypedependent transmembrane efflux pump (7). Overexpression of P-glycoprotein results in a reduction of intracellular concentrations of xenobiotics, drugs, and poisons, such as vinca alkaloids, anthracyclines, verapamil, colchicines, antimarialarials, and corticosteroids (2, 3, 8). P-glycoprotein is expressed on not only various types of tumor cells but also normal cells such as epithelial cells (9–11), CD34+ hematopoietic stem cells (12), and lymphocytes (13).

In various tumor cell lines, we and others have reported that transcription of MDR-1 is directly regulated by the human Y box-binding protein-1 (YB-1), an MDR-1 transcription factor, and that activation of YB-1 is induced in response to genotoxic stresses including multiple drugs (14, 15). In lymphocytes, our group reported previously that the transcription of MDR-1 gene and expression of P-glycoprotein are induced by soluble factors including lymphocyte-activation cytokines such as interleukin (IL)-2, resulting in the activation of YB-1 and induction of its binding to DNA (13).

In addition to soluble factors, intercellular adhesion or adhesion to the extracellular matrix transduces signals via functional molecules on lymphocytes and these processes are involved in the regulation of immunological reactions (16, 17). Hyaluronan, a representative component of extracellular matrix, is a sulfated glycosaminoglycan bearing linear repeats of disaccharide-β-D-glucuronyl-β-D-N-acetylgalcosamine (18).

The principal known receptor of hyaluronan is CD44 (19). CD44 is a 90-kDa transmembrane glycoprotein widely distributed on T lymphocytes, granulocytes, monocytes, fibroblasts, keratinocytes, and epithelial cells (20). Stimulation of CD44 with hyaluronan plays a role in various physiological functions, such as cell-cell adhesion, cell-substrate interactions, and lymphocyte recruitment, as well as pathological processes such as

* This work was supported by a research grant-in-aid for Scientific Research by the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the University of Occupational and Environmental Health, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MDR, multidrug resistance; YB-1, Y box-binding protein 1; IL, interleukin; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; CMV, cytomegalovirus; TBS, Tris-buffered saline; RA, rheumatoid arthritis.
chronic inflammation and metastasis of malignant cells (19). Hyaluronan is usually generated as a high molecular weight polymer, a native hyaluronan (18), but a fragmented hyaluronan, a digested low molecular weight oligomer, is detected in certain pathological conditions, such as inflammation and tumors (21, 22). Several studies have reported that the biological activities of fragmented hyaluronan markedly differ from native hyaluronan in endothelial cell, various malignant cells, and macrophages (23–26).

Although MDR-1 transcription in lymphocytes is induced by various activation stimuli such as cytokines, there is little information on whether stimulation with hyaluronan induces activation and MDR-1 transcription in resting CD4+ T cells. Furthermore, it is also not clear at present whether the effect of fragmented hyaluronan on activation and regulation of MDR1 are different from native hyaluronan in CD4+ T cells. The present study was designed to determine the effects of fragmented hyaluronan on CD4+ T cell activation and its role in P-glycoprotein-mediated multidrug resistance, particularly in fragmented hyaluronan-rich microenvironments such as areas of chronic inflammation or tumors.

**EXPERIMENTAL PROCEDURES**

*Isolation of Peripheral Blood Mononuclear Cells from Healthy Donors*—We isolated peripheral blood mononuclear cells (PBMCs) from healthy donors by density gradient centrifugation using Lymphocyte Separation Medium 50494 (GE Healthcare) as described previously (27, 28). We confirmed that purified PBMCs contained more than 90% of lymphocytes (CD4, CD8, or CD20 positive cells) and less than 10% of CD14 positive monocytes by immunostaining. The study was approved by the Human Subject Research Committee of the University of Occupational and Environmental Health, School of Medicine, and informed consent was obtained from all donors who enrolled in the study.

CD4+ T Cells Isolation from PBMCs—CD4+ T cells were purified by negative selection using magnetic beads according to the recommended procedure supplied by the manufacturer (CD4 negative isolation kit; Dynal Biotech, Japan). The purity of the CD4+ T cell subset was determined by flow cytometry to be greater than 90%.

Reagents and mAbs—Fragmented (1.7, 6.9, and 40 kDa) and native (950 kDa) hyaluronan were kindly donated by the Tokyo Research Institute of Seikagaku (Tokyo, Japan). The following monoclonal antibodies (mAbs) were used as purified Ig in preparation of staining and analysis of cell surface or cytoplasmic molecules, blocking of CD44, and Western blotting analysis. MRK16 (a specific mAb against P-glycoprotein; Kyowa Medex, Tokyo), a specific antibody against YB-1 (a binding protein to the Y box and CCAAT box, which is critical for the cis-regulatory element that regulates drug-induced MDR-1 gene expression (14)), CyChrome-conjugated CD4 mAb, CyChrome-conjugated CD8 mAb, phycoerythrin-conjugated CD69 mAb, fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab, fluorescein isothiocyanate-conjugated anti-rabbit IgG Ab (BD Biosciences), Hermes-1 (anti-human CD44 purified monoclonal antibody; Endogen), purified rat IgG₂a (anti-KLH, a control mAb for Hermes-1; BD Biosciences), and F4 (monoclonal anti-P-glycoprotein antibody; Sigma).

**Flow Cytometric Analysis**—Staining and flow cytometric analysis of PBMCs or CD4+ T cells were conducted by standard procedures as described previously using a FACScan (BD Biosciences) (27, 28). Briefly, PBMCs or CD4+ cells (2 × 10⁵ cells/well) were initially incubated with polyclonal γ-globulin (10 μg/ml, Yoshitomi Pharmaceutical Co.) for blocking of Fc receptors and then incubated with MRK-16, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG Ab in FACS medium consisting of phosphate-buffered saline, 0.5% human serum albumin, and 0.2% NaN₃ (Sigma). For the two-color analysis, we incubated PBMCs with CyChrome-conjugated CD4 mAb, CD8 mAb, or CD4+ cells with phycoerythrin-conjugated CD69 mAb after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibody two-color-stained cells were detected by electronic gating based on their CD4, CD8, or CD69 expression using a FACScan. Amplification of mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as reported previously (27–29). The data were used to construct the calibration curve of mean fluorescence intensity versus antibody binding capacity. The cell specimen was analyzed on the FACScan and the antibody binding capacity calculated by interpolation on the calibration curve. When the green fluorescence laser detection was set at 500 nm in the FACScan, the antibody binding capacity was equal to (202.98 × exp (0.0092 × mean fluorescence intensity), (R² = 0.9995)). Subsequently, the specific antibody binding capacity was obtained after correcting for the background, and apparent antibody binding capacity of the negative control anti-mouse IgG Ab. The specific antibody binding capacity is the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

**Immunostaining and Confocal Microscopy Analysis**—PBMCs or CD4+ T cells were plated onto a 12-well culture dish (2 × 10⁵ cells/well) and incubated for 60 min at 37 °C in the presence or absence of 50 μg/ml 6.9-kDa fragmented hyaluronan in RPMI 1640 containing 5% fetal calf serum. The cells were then treated with 4% formaldehyde (Sigma) in FACS medium for 15 min and then with 0.1% saponin (Sigma) in FACS medium. The obtained cells were incubated with a specific antibody against YB-1 for 30 min at 4 °C. Subsequently, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG Ab at saturating concentrations in FACS medium. We performed confocal analysis of YB-1 using a LSM 5-pascal inverted laser scan microscope (Carl Zeiss Microscope Systems, Germany).

**Gel Shift Assay**—Nuclear extracts from PBMCs were prepared as described previously (11) and then incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan. In the next step, 4 μg of nuclear protein were preincubated for 20 min at room temperature in 15 μl of buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4% glycerol, and 40 mM NaCl) containing 0.5 μg of poly(dI-dC) (GE Healthcare) and a ³²P-end-labeled double-stranded oligonucleotide containing the YB-1 consensus binding site (5’-GGGCAGTTTTAGCCA-
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The obtained cell fractions were melted with solvoue-350 and 10 ml of HIONIC-FLUOR (PerkinElmer) was added. The medium fractions were mixed with 10 ml of mixtures of tolulene (Wako, Osaka, Japan), methanol (Wako), ethyleneglycol mono-ethyl ether (Nacalai tesque), and PERMAFLUOR (Packard; mixture ratio 200:50:50:12). The radioactivity of each fraction was counted with a scintillation counter. The cell to medium ratio (C/M ratio), which is an index of intracellular and extra-cellular dexamethasone concentration ratio, was computed using the following formula: C/M ratio = ([3H in cell fraction/14C in cell fraction])/([3H in medium fraction/14C in medium fraction]).

CD44 Blocking Analysis—CD4+ cells were incubated with 10 µg/ml Hermes-1 or rat IgG2a as control mAb for 30 min at 37 °C prior to incubation with or without 50 µg/ml 6.9-kDa fragmented hyaluronan, the cells were used for the following experiments.

Western Blot Analysis—After incubation with or without 50 µg/ml 6.9-kDa fragmented hyaluronan, 2 × 10^6 of CD4+ T cells were homogenized in the presence of a hypotonic buffer (10 mM HEPE, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithio-orthoetol). The cells were allowed to swell on ice for 15 min, after which 0.5% (v/v) Nonidet P-40 was added and vortexed. The homogenate was centrifuged at 20,000 × g to obtain pellet containing membrane. The pellet containing equal amounts of proteins prepared from 2 × 10^6 of CD4+ T cells were subjected to SDS-PAGE. The separated proteins were transferred onto the nitrocellulose membrane (Schleicher & Schuell). After blocking with TBST-milk (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween 20, and 5% nonfat dry milk), the membrane was incubated overnight with 10 µg/ml F4 at 4 °C. The membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated anti-mouse mAb (1:1000 dilution in TBST) for 30 min at room temperature. After three washes in TBST, proteins were detected using a chemiluminescence reagent (Detection Reagent, Amersham Biosciences) following the procedure recommended by the manufacturer.

Statistical Analysis—Values are expressed as mean ± S.D. Student’s t-test was used to compare data between two groups. One-way analysis of variance and Bonferroni correction were used to compare data between three or more groups. p < 0.05 was considered statistically significant.

RESULTS

Fragmented Hyaluronan Up-regulates MDR-1 Gene Expression on PBMCs—We first assessed the effect of hyaluronan on P-glycoprotein expression in PBMCs. Expression of P-glycoprotein, as recognized by MRK16, was significantly induced by the addition of the 6.9-kDa fragmented hyaluronan during the observation periods (0–6 h), particularly at 6 h after incubation. In contrast, native hyaluronan (950 kDa) failed to induce P-glycoprotein expression on PBMCs (Fig. 1A). Therefore, we evaluated the expression of P-glycoprotein after a 6-h incubation with 6.9-kDa fragmented hyaluronan in the following studies. We observed that expression of P-glycoprotein was augmented in a dose-dependent manner up to 50 µg/ml 6.9-kDa fragmented hyaluronan (Fig. 1, B and C).
Fragmented Hyaluronan Induces Nuclear Translocation of YB-1 in PBMCs—MDR-1 gene expression is initiated by activation and nuclear translocation of YB-1 in human lymphocytes (12). We examined the intracellular distribution of the transcriptional factor YB-1 in PBMCs by immunostaining using anti-YB-1 monoclonal antibody. Confocal microscopic analysis showed localization of YB-1 in the cytoplasm of PBMCs at basal conditions and its translocation into the nucleus within 60 min of incubation with 6.9-kDa fragmented hyaluronan (Fig. 2A).

We confirmed the induction of the DNA binding activity of YB-1 by 6.9-kDa fragmented hyaluronan by electrophoretic mobility shift assay. Subsequently, we prepared nuclear extracts from PBMCs at basal conditions and after incubation with 6.9-kDa fragmented hyaluronan, and incubated them with a 32P-labeled oligonucleotide containing YB-1 consensus binding sites. We observed dense bands in the mixture of oligonucleotides and nuclear extracts derived from PBMCs stimulated with 6.9-kDa fragmented hyaluronan (Fig. 2B). These results imply that incubation of PBMCs with 6.9-kDa fragmented hyaluronan resulted in a sequence of events including activation of cytoplasmic nuclear factor YB-1, its nuclear translocation, and finally the binding of activated YB-1 to the promoter region of the MDR-1 gene. These results were consistent with those observed in the expression of P-glycoprotein in immunostaining.

YB-1 Antisense Inhibits P-glycoprotein Expression on Lymphocytes—To determine whether YB-1 is directly coupled with fragmented hyaluronan-induced MDR-1 gene activation, we assessed the expression of P-glycoprotein on PBMCs transfected with YB-1 antisense expression plasmid (PRC/CMV AS) or control vacant vector and compared the levels of P-glycoprotein expression on PBMCs incubated with or without 6.9-kDa fragmented hyaluronan. In comparison with the vector alone, introduction of YB-1 antisense significantly reduced the expression of P-glycoprotein on PBMCs. Fragmented hyaluronan stimulation significantly increased the expression of P-glycoprotein on control cells, but such induction was abolished by transfection of YB-1 antisense (Fig. 3).

Fragmented Hyaluronan Up-regulation of P-glycoprotein Expression on CD4+ T Cells—To investigate the expression of P-glycoprotein on lymphocytes in more detail, we next performed two-color analysis using anti-CD4 and -CD8 antibodies and examined P-glycoprotein expression on each subset of lymphocytes. Flow cytometric analysis showed that P-glycoprotein expression was significantly augmented by 6.9-kDa fragmented hyaluronan on CD4+ but not CD8+ cells in PBMCs (Fig. 4A). Therefore, in further studies, we analyzed purified CD4+ T cells...
the addition of the fragmented hyaluronan (Fig. 4, A–D). In the present study, we evaluated whether up-regulation of P-glycoprotein by the 6.9-kDa fragmented hyaluronan occurs exclusively on CD69 high expressing CD4+ T cells.

Expression of P-glycoprotein Induces Excretion of Intracellular Dexamethasone in CD4+ T Cells—To investigate the association between expression of P-glycoprotein on CD4+ T cell and exclusion of drugs through P-glycoprotein, the intracellular and extracellular concentration of dexamethasone was determined. Stimulation with fragmented hyaluronan resulted in a significant decrease of intracellular dexamethasone of CD4+ T cells (Fig. 6). To confirm the functional involvement of P-glycoprotein in the decrease of intracellular dexamethasone, we added 100 ng/ml cyclosporin A, a competitive inhibitor of P-glycoprotein, to fragmented hyaluronan-stimulated CD4+ T cells. Excretion of dexamethasone in CD4+ T cells was completely inhibited by 100 ng/ml cyclosporin A (Fig. 6).

Anti-CD44 mAb Hermes-1 Inhibits Up-regulation of MDR-1 Gene Expression in CD4+ T Cells—One of the principal cellular receptors for hyaluronan is CD44 (19). Therefore, we used Hermes-1, a mAb blocking CD44, to investigate whether the CD44s epitope was essential for up-regulation of MDR-1 gene expression in CD4+ T cells by fragmented hyaluronan. We found that fragmented hyaluronan markedly induced the nuclear translocation of YB-1 and the expression of cell surface P-glycoprotein by immune staining and Western blotting analysis. The inducibility was abolished by preincubation with Hermes-1, confirming the involvement of signal thorough CD44 molecules in fragmented hyaluronan-induced P-glycoprotein expression (Fig. 7).

**DISCUSSION**

In terms of interaction of hyaluronan with cell surface CD44, three functional categories of CD44 have been reported: non-binding CD44, non-binding CD44 unless activated, and constitutively active CD44. Most cells of connective tissue, as well as some proliferative epithelial cells and tumor cells appear to express constitutively active CD44. In contrast, CD44 is functionally inactive on resting T cells, and is not able to bind hyaluronan without external stimuli such as TCR stimulation (37). Fragmented hyaluronan but not native high molecular weight hyaluronan has been shown to stimulate the expression of cytokines, proinflammatory chemokines, and cell adhesion molecules possibly through a mechanism involving CD44 in endothelial cells, various malignant cells, and macrophages (24–26).

In this report, we demonstrate a novel physiological function of fragmented hyaluronan on CD4+ T cells. The main findings of the present study were as follows. 1) Fragmented hyaluronan, but not the native high molecular weight hyaluronan, was able to induce transcriptional regulation of the MDR-1 gene, YB-1 activation followed by P-glycoprotein expression, in CD4+ T cells. 2) Expression of P-glycoprotein on CD4+ T cells induced by fragmented hyaluronan was observed preferentially on activated CD4+ T cells. 3) Overexpression of P-glycoprotein resulted in dexamethasone excretion from CD4+ T cells, which was inhibited by the competitive inhibitor cyclosporin A. 4) Transcriptional induction of MDR-1 gene by fragmented

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hyaluronan (Fig. 5, E and F). These results imply that expression of P-glycoprotein is closely associated with activation of CD4+ T cells and that up-regulation of P-glycoprotein by the 6.9-kDa fragmented hyaluronan occurs exclusively on CD69 high expressing CD4+ T cells.

**FIGURE 3. YB-1 antisense inhibits fragmented hyaluronan-induced P-glycoprotein expression on PBMCs.** Flow cytometric analysis showed P-glycoprotein expression on 2 × 10⁶ normal PBMCs that were transfected with YB-1 antisense constructs (closed bars) or control vacant vector (open bars) and then incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan (Fr. HA). Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of five independent experiments. Statistical analysis was performed using the paired t test. *, p < 0.05; **, p < 0.01.
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Hyaluronan was mediated through CD44 molecules on CD4+ T cells.

Other investigators have reported that fragmented hyaluronan binding to cell surface CD44 progressively increased in avidity with increasing oligomer size from 20-mers to 38-mers, and that fragmented hyaluronan (38), especially the 6.9-kDa fragmented hyaluronan (36-mers), enhanced CD44 cleavage as well as promoted tumor cell mobility without certain stimuli (e.g. phorbol ester and anti-CD44 stimulating antibodies in vitro), whereas a large polymer hyaluronan failed to induce them (39). This evidence was consistent with our results showing that 6.9-kDa fragmented hyaluronan induced expression of P-glycoprotein on lymphocytes most strongly.

In contrast to our results, Misra et al. (40) reported that fragmented hyaluronan reduces MDR-1 expression in human breast carcinoma cells. The reasons for the conflicting result on the function of fragmented hyaluronan are due, at least, to the difference of oligomer size of fragmented hyaluronan. They used oligomers of 3–10-mers that could do only monovalent binding, whereas we used oligomers of 36-mers, which allow at least divalent binding (38). The oligomers of 3–10-mers antagonize constitutive interaction between hyaluronan polymer and CD44 by competitively replacing, because multivalent interaction can transduce signals although a monovalent interaction does not (40). On the other hand, we showed that the oligomers of 36-mers induce signal transduction after interaction with CD44 molecules on the resting CD4+ T cells and that the interaction induces activation of CD4+ T cells and expression of MDR1 mRNA. Others reported that 4–6-mers induced maturation of dendritic cells without CD44 (41). Therefore, different sizes of hyaluronan oligomers might induce the different effects on CD44-hyaluronan interaction.

Therefore, hyaluronan changes activities of MDR-1 induction, as well as other biological activities, depending on its molecular weight. The oligomers of 3–10-mers (probably 4–6mers, 0.8–1.2 kDa) inhibit MDR-1 expression competitively by monovalent interaction with CD44. The larger oligomers (~1.7 kDa), small and intermediate sized polymers (~500 kDa) could induce MDR-1 expression by more than the divalent interaction with CD44, and the oligomers of 36-mers (6.9 kDa) might be the most potent activators of MDR-1 expression. The large sized polymers (probably ~600 kDa) could not induce MDR-1 expression.

Furthermore, the manner of hyaluronan production is different between resting CD4+ T cells and human breast carcinoma cells. Although CD4+ T cells constitutively express mRNA for hyaluronan synthases, their surface hyaluronan expression becomes detectable only after cell activation. In contrast, human breast carcinoma cells spontaneously produce hyaluronan without any stimulation, express constitutively active CD44, but CD44 on resting T cells is functionally inactive and cannot bind hyaluronan without external stimuli such as TCR stimulation (37). Thus, unless CD4+ T cells are stimulated, they express neither MDR-1, the active form of CD44, nor cell surface hyaluronan, whereas on human breast carcinoma cells MDR-1 could be constitutively expressed through endogenous small and intermediate sized hyaluronan polymers by CD44-mediated self-activation. We, therefore, document that 6.9-kDa fragmented hyaluronan (36-mers) interacts with CD44 on the resting CD4+ T cells, and that the interaction induces activation of CD4+ T cells and induction of MDR-1 expression.

Fragmented hyaluronan can be generated in vivo by various mechanisms, such as enzymatic digestion by hyaluronidases and acid hydrolases, degradation by oxygen-derived free radi-
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FIGURE 5. Fragmented hyaluronan induces activation of CD4+ T cells, which results in the expression of P-glycoprotein. A–C, flow cytometric analysis showed increased expression of CD69 on 5 × 10⁵ of CD4+ T cells isolated from PBMCs (A and gray area in C) at 3 h after stimulation with 50 μg/ml 6.9-kDa fragmented hyaluronan (B and solid black line in C). The region R1 indicates CD69 high expressing cells. D, data represent the percentage of CD69 high expressing cells among 5 × 10⁵ of CD4+ T cells isolated from PBMCs from 10 independent donors incubated with (solid bars) or without (open bars) 50 μg/ml 6.9-kDa fragmented hyaluronan for 3 h. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired t test. *, p < 0.05. E, flow cytometric analysis showed P-glycoprotein expression on CD69 high or low expressing CD4+ T cells after a 6-h incubation with or without 50 μg/ml 6.9-kDa fragmented hyaluronan (Fr. HA). F, P-glycoprotein expression on CD69 high or low expressing CD4+ T cells after a 3-h incubation with (closed bars) or without (open bars) 50 μg/ml 6.9-kDa fragmented hyaluronan (Fr. HA). Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired t test. *, p < 0.05.

cals, or de novo synthesis of low molecular mass hyaluronan (46–47). Fragmented hyaluronan exists in the place of tumor invasion or inflammation, and closely relates to their pathogenesis (21–24).

In rheumatoid arthritis (RA), which is characterized by progressive polyarthritis with occasional extraarticular involvement, fragmented hyaluronan is increased in the RA synovium and synovial fluid (21, 48). Although the production of native hyaluronan by RA synovial cells is promoted according to the aggravation of inflammation by inflammatory cytokines including IL-1β and tumor necrosis factor-α (49), the activity of hyalurondase produced by CD14+ monocytes is so high that native hyaluronan is digested to fragmented hyaluronan immediately at the loci of inflammation (50). Furthermore, the production of fragmented hyaluronan is accelerated due to oxygen-derived free radicals in the inflammation locus like synovitis (21, 48). Our previous studies showed that fragmented hyaluronan, which is increased by inflammation in proportion to RA disease activity, induced the expression of vascular cell adhesion molecule-1 on synovial cells and is involved in the pathogenesis of RA (51, 52).

Recently, we found that IL-2, a potent stimulus of lymphocytes (53, 54), up-regulated P-glycoprotein expression on lymphocytes via activation of transcriptional factor YB-1 and that such up-regulation markedly reduced intracellular corticosteroid concentration in vitro (13). In this study, we found that 6.9-kDa fragmented hyaluronan was also a potent stimulus of CD4+ T cells as well as IL-2. We demonstrated that 6.9-kDa fragmented hyaluronan could activate resting CD4+ T cells and induce P-glycoprotein expression simultaneously without any other co-stimuli.

We propose that the expression of P-glycoprotein in accordance with activation of CD4+ T cells is a serious problem in autoimmune diseases including RA and systemic lupus erythematosus. Autoimmune diseases are characterized by inflammation induced by activation of autoreactive T cells and production of autoantibodies from activated B cells, which is enhanced by intercellular adhesion, adhesion to extracellular matrix, and cytokines such as IL-2 (51–55). The main strategy for treatment is to control such autoreactive lymphocytes with corticosteroids, disease-modifying antirheumatic drugs, and other immunosuppressants (56). Corticosteroids, certain immunosuppressive agents, and disease-modifying antirheumatic drugs, including chloroquine and sulfasalazine, are extruded from lymphocytes by P-glycoprotein (3, 13, 57). We note that P-glycoprotein acts as a “hydrophobic vacuum cleaner,” i.e., P-glycoprotein catches drugs like a vacuum cleaner when they pass through the cell membrane, and then releases them outside the cell. Substrates of P-glycoprotein, including corticosteroids and some disease-modifying antirheumatic drugs, cannot reach the cytoplasm by increasing the number of P-glycoproteins on lymphocytes, thus resulting in treatment failure. Indeed, our group and others have reported that low cytoplasmic corticosteroid concentrations, caused by increased P-glycoprotein-mediated efflux of corticosteroids from lymphocytes, is one of the mechanisms of corticosteroid resistance in inflammatory bowel disease, asthma, and systemic lupus erythematosus (58–60). Furthermore, the expression of P-glyco-
protein on lymphocytes and the efficacy of cyclosporin A, a competitive inhibitor of P-glycoprotein, have been reported in refractory RA patients (61–63).

As another pathological relevance of hyaluronan, several studies have shown that fragmented hyaluronan strongly stimulates tumor invasion, growth, and P-glycoprotein-mediated multidrug resistance (22, 25, 40). Otherwise, our results suggest that the fragmented hyaluronan around tumors activates not only tumors but also CD4+ T cells of the host. Expression of P-glycoprotein on CD4+ T cells is induced in accordance with activation of CD4+ T cells by fragmented hyaluronan around tumors (64), and then these cytokines might activate effector cells including LAK and NK resulting in enhancement of the anti-tumor effect (65). Furthermore, CD4+ T cells could survive by acquiring P-glycoprotein-mediated chemotherapy resistance. Thus, it will be advantageous for the host that expression of P-glycoprotein on CD4+ T cells is increased in accordance with activation of CD4+ T cells induced by fragmented hyaluronan around tumors.

In this study, we propose that the sequential events in fragmented hyaluronan-stimulated CD4+ T cells, consist of CD44 activation, MDR-1 transcription, P-glycoprotein expression, and production of P-glycoprotein substrates that can be inhibited by P-glycoprotein competitors. Our findings suggest that a characteristic change of extracellular matrix in a specific environment could directly modulate the immune system. Our findings also expand our understanding of the interaction between tumors, chronic inflammation, and the immune system.

Acknowledgment—We thank T. Adachi for the excellent technical assistance.

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Fragmented Hyaluronan Induces Transcriptional Up-regulation of the Multidrug Resistance-1 Gene in CD4+ T Cells
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doi: 10.1074/jbc.M601030200 originally published online October 12, 2006

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