Enhanced Production of Interleukin 6 in Peripheral Blood Monocytes Stimulated with Mucins Secreted into the Bloodstream

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Abstract  Purpose: It has been reported that tumor progression is correlated with the serum level of interleukin 6 (IL-6). The purpose of this study was to investigate by what mechanism, other than production from tumor cell, the serum level of IL-6 is elevated in the tumor-bearing state.  Experimental Design: Monocytes from healthy donors were cultured in the presence of sera from colon cancer patients, and the activity to elevate IL-6 production was estimated. This activity of serum was also examined after various biochemical treatments.  Results: When monocytes from healthy donors were cultured in the presence of sera from patients with colon cancer, secretion of IL-6 from the cells was markedly elevated. Serum proteins were fractionated on Sepharose 4B and the activity to elevate IL-6 production was found in the excluded fractions. Sialyl Tn antigen was detected in these same fractions. By excluding some mucins from the serum, the inducing activity was reduced to 40% of the original level. Furthermore, we purified mucins from the conditioned medium of colon cancer cells. Production of IL-6 was effectively elevated by a small amount of purified mucins in a dose-dependent manner. When the inducing activity was examined in the presence of binding or competitive inhibitors to the scavenger receptor, the effect was remarkably reduced.  Conclusions: Mucins secreted from colon cancer cells into the bloodstream induce production of IL-6 in peripheral blood monocytes through the scavenger receptor, which may be responsible for the high level of serum IL-6 in colon cancer patients.

It is generally agreed that malignant cells are able to escape immune surveillance. The antitumor immune response is regulated by several factors, including cytokines produced by the tumor and other cells of the tumor stroma. It seems likely that the local cytokine microenvironment, acting on the tumor cell or on the adjacent cells, can either block or facilitate tumor growth, and that proinflammatory cytokines strongly influence the immunologic state. Interleukin 6 (IL-6) is a pleiotropic cytokine, originally identified as a differentiation factor for B lymphocytes, which is produced by a variety of cells including monocytes, endothelial cells, fibroblasts, and some types of tumor cells (1). High levels of IL-6 have been found in ascites and serum of patients with ovarian cancer (2) and in serum of colon cancer patients (3, 4). An elevated level of serum IL-6 is associated with a poor prognosis for patients with ovarian cancer (2, 5). Patients with a high level of serum IL-6 have a significantly shorter time to disease progression, and their overall survival time is reduced (6). Thus, it seems that IL-6 is involved in malignant transformation and tumor progression (7–13). In particular, IL-6 seems to play a crucial role in colon cancer (14–16). Recently, it has also been reported that IL-6 is secreted from macrophages when they are cocultured with colon cancer cells, but not with lung, prostate, or cervical cancer cells. The IL-6 secretion is followed by IL-6–induced IL-10 production by colon cancer cells in a paracrine fashion (17). These reports are consistent with the fact that circulating levels of IL-10 and IL-6 are elevated in colon cancer patients compared with controls (3, 4). IL-6 gene expression is modulated by inflammatory stimuli such as lipopolysaccharide, various viruses, and a variety of cytokines. Some reports have investigated mechanisms of monocyte/macrophage activation in tumor tissues (18, 19), but few studies have been focused on how monocytes/macrophages can respond to cancer cells directly and/or to products secreted from cancer cells indirectly. In the present study, we found an interesting mechanism causing elevation of serum IL-6, in which mucins secreted from cancer cells are responsible for the production of IL-6 in peripheral blood monocytes.
It is well known that many tumors arising from epithelial tissues produce mucins. Mucins are a heterogeneous family of high molecular mass proteins that are broadly defined by their high content of carbohydrates, which are mainly composed of O-linked sugars. On malignant transformation, many epithelial cells produce mucins in abnormal amounts and/or with abnormal glycosylation patterns (20). Mucins produced by tumor cells are found in the sera of cancer patients and are used as disease markers. It has been reported that patients with higher levels of serum mucins have a lower 5-year survival rate (21). However, little is known about the biological significance of serum mucins. Previously we showed that mucins produced by a human colon cancer cell line, LS 180, could induce cyclooxygenase-2 (COX-2) in monocytes/macrophages through the scavenger receptor, resulting in enhanced production of prostaglandin E2 (PGE2; ref. 22). It has been reported that the scavenger receptor could recognize a pattern of anionic charges on molecules such as acetyl low-density lipoprotein, poly I, and fucoidan, and that the binding site of the receptor consists of a Lys/Arg cluster (23). Anionic charges due to sialic acid and sulfate borne on O-glycans of mucins may be involved in this recognition.

It has been reported that IL-6 gene expression is up-regulated on activation of the signaling pathway involving protein kinase C and/or cyclic AMP–dependent protein kinase (24–26). Binding of ligands to the scavenger receptor also activates the protein kinase C–dependent pathway (27). Thus, it seems possible that binding of mucins to the scavenger receptor could enhance the production of IL-6. In the present study, we show that mucins secreted into the bloodstream of patients enhance the production of IL-6 in peripheral blood monocytes, probably resulting in high levels of serum IL-6 followed by induction of IL-10 production in colon cancer cells.

Materials and Methods

Materials. Peripheral blood was obtained from 10 colon cancer patients (four male, six female; age 59 to 88 years, mean age 70; stage III: 4 cases, stage IV: 6 cases) and seven healthy adult volunteers in accordance with a protocol approved by the Kansai Medical University committee. Patients with acute infection, chronic inflammatory disease, myocardial infarction, or uremia were excluded. Peripheral blood monocytes were separated by Ficoll-Isopaque (Amersham Bioscience, Uppsala, Sweden) centrifugation according to the instructions of the manufacturer. The cells were suspended in RPMI 1640 supplemented with 10% FCS and allowed to attach to the culture plate for 1 hour. Nonadherent cells were removed by washing the plate with RPMI 1640, and adherent cells were collected. A murine anti–sialyl Tn monoclonal antibody (mAb), designated as MLS 132, was prepared as previously described (28). MLS 132 mAb– or control immunoglobulin G–conjugated Sepharose 4B and incubated for 4 hours. The culture medium was collected and IL-6 levels were assayed.

Fractionation of serum proteins on Sepharose 4B and dot blot analysis. Serum samples from colon cancer patients were subjected to gel filtration on Sepharose 4B (1 × 110 cm) and then eluted with 25 mmol/L sodium phosphate buffer (pH 7.5) and 0.15 mol/L NaCl. Fractions of 1.2 mL were collected and absorbance at 280 nm was determined. One hundred microliters of each fraction were loaded on a nylon membrane and sialyl Tn antigen borne on the mucin core protein was detected by using MLS 132 mAb.

Assay of interleukin-6 activity. The level of IL-6 was determined by ELISA. To the culture medium of healthy monocytes (1 × 10⁶ cells), various samples containing mucins were added and cultured in RPMI 1640 supplemented with 10% FCS for the time indicated in the figure legend. The culture medium was collected and IL-6 levels were assayed.

Removal of mucins bearing a sialyl Tn antigen from serum. Serum obtained from cancer patients was mixed with MLS 132 mAb or control immunoglobulin G–conjugated Sepharose 4B and incubated for 4 hours. After centrifugation, the supernatant was used in assays to determine its effect on induction of IL-6 in monocytes, as described above.

Effect of binding inhibitors to the macrophage scavenger receptor. Monocytes (1 × 10⁶ cells) from healthy donors were stimulated with the serum from cancer patients in the presence of a peptide (10 µg/mL) corresponding to the binding site for ligands of the macrophage scavenger receptor or sulfatide (10 µg/mL), and IL-6 secreted into the medium was determined as described above.

Results

In accordance with its potential role in cancer progression, an elevated serum level of IL-6 has been reported in cancer
patients, including colorectal cancer patients. As expected, the IL-6 level in serum of colon cancer patients was significantly higher (range, 38-80 pg/mL; mean, 58.7 pg/mL) than that of healthy donors (0-9 pg/mL; Fig. 1A). High levels of serum IL-6 were frequently observed, irrespective of the different potentials of various tumor cells to produce IL-6. To address this issue, we examined whether or not IL-6 is induced in monocytes obtained from colon cancer patients. Total RNA was isolated from monocytes and reverse transcription-PCR was carried out as described in Materials and Methods. IL-6 mRNA was detectable in more than 60% of the monocyte samples obtained from the patients, as shown in Fig. 1B. Therefore, in other patients with a detectable level of serum IL-6, the tumor itself and/or other host cells may have produced the IL-6.

Previously, we showed that mucins secreted from colon cancer cells activated monocytes/macrophages through a scavenger receptor, resulting in induction of COX-2 mRNA and elevated production of PGE$_2$ (22). We also found that mucins from the bloodstream of patients with gastrointestinal cancer induced COX-2 mRNA in monocytes.$^5$ Furthermore, we examined whether or not mucins may be one of the factors that enhance the production of IL-6 in monocytes. Monocytes were obtained from a healthy donor, cultured in the presence of serum (0.5 mL) prepared from cancer patients, and then the level of IL-6 in the culture medium was determined by ELISA. As shown in Fig. 2, 60% of patient serum samples induced a remarkable enhancement of IL-6 production, but most of the sera from healthy donors showed no effect. Mean levels of IL-6 secreted from monocytes treated with patient and control sera were 146 and 32 pg/mL, respectively ($P < 0.05$). These results suggest that some factors to enhance the production of IL-6 are contained in the serum.

To further examine the production of IL-6 in response to serum, monocytes obtained from a healthy donor were cultured in the presence of various amounts of serum or for various time intervals. As shown in Fig. 3A and B, production of IL-6 in monocytes was related to the amount of serum added, and up-regulation was observed as early as 1 hour after the incubation commenced. It is generally agreed that production of IL-6 is up-regulated by various stimuli such as lipopolysaccharide, IL-1, and platelet-derived growth factor. Because mucins are glycoproteins with extremely high molecular weights, serum from a colon cancer patient was fractionated on Sepharose 4B. One hundred microliters of each fraction were added to the culture medium of monocytes. The excluded fractions remarkably elevated the secretion of IL-6, as shown in Fig. 4A. Sialyl Tn antigen was also detected in these fractions (Fig. 4B). To further confirm the effect of mucins, we purified mucins from the culture medium of the colon cancer cell line LS 180 as previously described (22). Production of IL-6 was elevated effectively by purified mucins in a dose-dependent manner (Fig. 4C). We also examined the production of IL-6 in response to serum after excluding mucins bearing a sialyl Tn antigen. The serum was mixed with anti–sialyl Tn antigen– or with a control mAb–conjugated Sepharose and was then subjected to centrifugation. The resultant supernatant was added to the culture medium of healthy monocytes, followed by incubation for 24 hours. The level of IL-6 in the culture medium was reduced to about 40% of the original level by

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$^5$ Yokoigawa et al., unpublished data.
excluding the mucins bearing the sialyl Tn antigen, clearly indicating that the mucins were responsible for more than half of the activity to enhance IL-6 production (Fig. 5).

Previously, we showed that binding of mucins to monocytes was inhibited by poly I, but not by poly C, suggesting that the macrophage scavenger receptor is responsible for the binding to the mucins. It has been reported that the scavenger receptor could recognize a pattern of anionic charges on molecules such as acetyl low-density lipoprotein, poly I, fucoidan, and lipopolysaccharide through a lysine and/or arginine cluster located on the collagen-like domain of the scavenger receptor. Thus, we examined the effect of other ligands to the scavenger receptor. Monocytes were incubated with bovine submaxillary mucin (100 μg/mL) or poly I (100 μg/mL) for 24 hours and IL-6 secreted from the cells was determined. As expected, these ligands of the scavenger receptor showed a similar activity to enhance the production of IL-6 (data not shown). We next examined the effect of sulfatide, which is one of the binding inhibitors, and a peptide corresponding to the site with a lysine cluster on the human scavenger receptor. When monocytes were incubated with the serum of cancer patients in the presence of sulfatide or the peptide, the production of IL-6 clearly decreased, indicating that monocytes were stimulated by mucins through the scavenger receptor (Fig. 6).

Discussion

Many immune evasion mechanisms are acquired by tumors as they progress from an early preneoplastic lesion to a fully malignant cancer, and cytokines play important roles in this process. Despite much evidence that IL-6 is associated with an adverse prognosis for many patients with cancer, the mechanism by which IL-6 production is induced has not been elucidated in detail. Recently, we showed that mucins secreted from colon cancer cells could induce COX-2 in monocytes/macrophages through a scavenger receptor (22) and that COX-2 was actually induced in monocytes of patients with mucins in their sera. Hsu et al. (27) reported that ligand binding to the macrophage scavenger receptor activates human THP-1 cells through a protein kinase C–dependent pathway, suggesting that IL-6 production may be induced in monocytes with mucins through a similar pathway. Therefore, we attempted to see if IL-6 production is actually induced in monocytes of patients with mucins in their sera. In spite of different levels of IL-6 production by the tumor cells themselves, production of IL-6 was enhanced by adding serum of cancer patients to the culture medium of monocytes.

When mucins purified from the culture medium of LS 180 cells were added to the culture medium of the monocytes, production of IL-6 was elevated in a dose-dependent manner. LS 180 cells produce MUC2 mucin, which is known to have a high number of tandem repeats (30). If a binding site for the receptor is present in each repeat unit, mucins have so many binding sites that many receptors could be bridged on the cell surface, probably resulting in potent signal transduction. To

Fig. 4. Elevated production of IL-6 in monocytes after treatment with mucins. Serum of a patient (1.5 mL) was subjected to gel filtration on Sepharose 4B column (1 x 110 cm) and then eluted with 25 mmol/L sodium phosphate buffer (pH 7.5) and 0.15 mol/L NaCl. A, fractions of 1.2 mL were collected and absorbance at 280 nm was determined. The proteins contained in 200 μL of each fraction were precipitated with ethanol. The precipitate was added to the culture medium of monocytes (1 x 10^6 cells). After 42 hours, secreted IL-6 was assayed as in Fig. 2. B, 100 μL of each fraction were loaded on a nylon membrane. Dot blot analysis was done using mAb MLS132. C, healthy monocytes (1 x 10^6 cells) were cultured for 24 hours in the presence of mucins purified from the culture medium of LS 180 cells, and secreted IL-6 was assayed by ELISA.

Fig. 5. Production of IL-6 by healthy monocytes stimulated with serum treated with anti–sialyl Tn antibody–conjugated Sepharose. Serum obtained from a patient with colon cancer was mixed with anti–sialyl Tn antibody– or control immunoglobulin G–conjugated Sepharose 4B and incubated at 4°C for 2 hours. After centrifugation, the resultant supernatant corresponding to 0.5 mL of serum was added to the culture medium of healthy monocytes, cultured for 24 hours, and then secreted IL-6 was assayed by ELISA. A, serum treated with control immunoglobulin G–Sepharose; B, serum treated with anti–sialyl Tn antigen mAb–Sepharose.
Overproduction of IL-6 in Mucin-Stimulated Monocytes

Further confirm the relationship between mucins and IL-6 production, the IL-6–inducing activity was examined after the removal of mucins bearing the sialyl Tn antigen from serum. The sialyl Tn antigen is highly expressed in various epithelial cancer tissues. Thus, anti–sialyl Tn mAb seems to be effective in removing mucins from the serum. The effect of this mucin-depleted serum on the production of IL-6 was remarkably reduced, indicating that mucins play a major role in the induction of IL-6 mRNA in monocytes. In addition, the production of IL-6 was enhanced within 1 hour, suggesting that it was enhanced directly in response to mucins, rather than indirectly via PGE2 or an unknown factor. In the later phase, however, there is a possibility that IL-6 production may be further elevated by the stimulation with PGE2. These results are consistent with the fact that although breast carcinomas contain very few mononuclear cells expressing cytokine mRNA, mucin-producing breast carcinomas exceptionally contain many lymphoid cells positive for cytokine mRNA in the tumor stroma (31).

In a previous article (22), we showed that the binding of mucins to monocytes was inhibited by poly I, fucoidan, and bovine submaxillary mucin, but not by poly C, suggesting that the macrophage scavenger receptor was responsible for the binding of mucins. It has been reported that the scavenger receptor could recognize a pattern of anionic charges on molecules such as acetyl low-density lipoprotein, poly I, and fucoidan (32, 33) and that the binding site of the receptor consists of a Lys/Arg cluster. As expected, other ligands such as poly I also enhanced IL-6 production (data not shown). We also examined the effect of sulfatide, which is known to bind to the scavenger receptor (34), and of a peptide corresponding to the Lys cluster of the human scavenger receptor. In the presence of these substances, production of IL-6 in monocytes was clearly decreased, indicating that production of IL-6 was enhanced through the scavenger receptor in monocytes. Because sulfatide binds to the receptor monovalently, its signal transduction may be so weak that its binding could inhibit the strong signal transduction induced by multivalent ligands such as mucins. Becker et al. (35) reported that tumor-infiltrating T-cell–derived IL-6 acts as a tumor growth factor in colon cancer. As described above, monocytes/macrophages were activated by mucins through the scavenger receptor. Because T cells do not express the scavenger receptor, IL-6 production by T cells is not induced at least by the same mechanism. Recently, it was reported that colon cancer cells initially promote IL-6 production by macrophages, which activate tumor cells to produce IL-10 (17). Thus, our results are consistent with the report that serum levels of IL-10 were high in colon cancer patients (3). Taken together, we suggest the following mechanism of interactions between infiltrated macrophages and cancer cells in colon cancer tissues. Macrophages are activated by mucins produced by colon cancer cells to secrete IL-6. IL-6 then stimulates the colon cancer cells to produce IL-10, which contributes to the evasion of antitumor activity by tumor-associated macrophages. Because IL-10 exhibits various immunosuppressive effects in vivo and seems to play a crucial role in colon cancer, high levels of serum IL-6 also have various effects, such as promotion of angiogenesis and impairment of host immune responses (36). In a previous article (22), we showed that COX-2 mRNA was induced in monocytes by mucins, leading to overproduction of PGE2. It is well known that overexpression of COX-2 and the subsequent overproduction of PGE2 have various biological effects such as enhanced expression of Bel-2 (37, 38), inhibition of immune surveillance (39), promotion of tumor angiogenesis (40, 41), and activation of matrix metalloproteinase-2 (42). Collectively, these findings suggest that mucins could promote favorable conditions for cancer cell growth in epithelial cancer tissues. In addition, Barnd et al. (43) showed that epithelial mucin is a human tumor antigen recognized by T cells. Thus, it has been considered that cancer vaccines to generate protective immunity may be useful in the clinic. Based on our finding that mucin is one of the factors to produce immunosuppressive cytokines from monocytes/macrophages, induction of an anti-mucin response may be effective in the prevention of immunosuppression in cancer patients.

References

Fig. 6. Inhibition of IL-6 production by a sulfatide or by a peptide corresponding to the Lys cluster of the macrophage scavenger receptor. In addition to the serum of a colon cancer patient, a sulfatide (10 μg/mL) or a peptide (10 μg/mL) was added to the culture medium of healthy monocytes (1 × 10⁶ cells), cultured for 24 hours, and then the IL-6 produced was assayed by ELISA. A, serum; B, serum plus sulfatide; C, serum plus peptide; D, RPMI culture medium.

www.aacrjournals.org Clin Cancer Res 2005;11(17) September 1, 2005 6131


Clinical Cancer Research

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