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A High Concentration of MMP-2/Gelatinase A and MMP-9/Gelatinase B Reduce NK Cell-mediated Cytotoxicity against an Oral Squamous Cell Carcinoma Cell Line

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Abstract. *Background: Recent studies have shown that matrix metalloproteinases (MMPs) from tumors influence the host immune system to reduce antitumor activity. The aim of this study was to examine the influence of MMP-2 and MMP-9 on the natural killer (NK) cell. Materials and Methods: NK cells were pretreated with either MMP-2 or MMP-9 in the experimental group but not in the control group. NK cell cytotoxicity against oral squamous cell carcinoma cells (OSCC) were examined using the [Cr^{51}] release assay and the expression levels of surface receptors on NK cells were measured by flow cytometry. Results: NK cell cytotoxicity was significantly reduced in the experimental group compared to the control group. Significant increases in KIR expression and decreases in NKG2D expression on the NK cells were observed in the experimental group. Conclusion: This study suggests an additional role of MMP-2 and MMP-9 in an immune escape mechanism of OSCC.*

Oral squamous cell carcinoma (OSCC) is characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes (1). Like other malignancies, the biology of OSCC is closely related to the host immune system. It has been shown that OSCC patients with more infiltration of host immune cells around tumors had better prognoses (2). Furthermore, OSCC patients in an advanced state of the disease had generally reduced host immune function (3, 4). Nevertheless, the exact role of immune cells, and of the immune system in general, in the development of OSCC and in tumor progression remains ambiguous. While the initiation of the process of tumorigenesis is clearly linked to carcinogens (*i.e.* tobacco or alcohol), its progression through a series of discrete genetic changes results in the emergence of a tumor that is resistant to immune effector cells (5, 6).

Natural killer (NK) cells, a type of effector cell of the human immune system, have a cytotoxic activity, which does not require prior sensitization, against malignant cells and virally infected cells. NK cells contribute significantly to immune surveillance by recognizing and destroying malignant tumor cells that evade antigen-specific detection by T-lymphocytes (7). It has been reported that the effector functions of NK cells are controlled by interactions involving specific NK cell receptors and their cognate ligands, either on target cells, or on other cells of the immune system (8, 9). A wide variety of NK cell activating-receptors or NK cell-inhibiting receptors such as NKG2D, CD94, and killer cell immunoglobulin (Ig)-like receptors (KIR) exist, many of which have short cytoplasmic domains and interact with transmembrane signaling adaptor molecules to activate or inhibit NK cell function (9). Hence, it is highly probable that these factors may be influenced by intrinsic or extrinsic proteinases, such as matrix metalloproteinases (MMPs) expressed on or secreted by a tumor.

MMPs are a family of Zn^{++}-dependent proteinases that can degrade all components of the extracellular matrix (ECM) (10). This family of endopeptidases is associated with ECM degradation in physiological and pathological conditions such as embryonic development, wound healing, angiogenesis, arthritis, inflammation and tumor metastasis (11, 12). MMPs are produced by cancer cells directly or through the induction of MMP synthesis by surrounding stromal cells. To successfully

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establish metastasis, the first step is the active migration of cancer cells from their tissue of origin, and involves the degradation of the underlying basement membrane which is made up of matrix macromolecules such as type IV collagen, laminin, and heparan sulfate proteoglycans. Thus the gelatinases (MMP-2 and MMP-9) are thought to play an important role in basement membrane degradation because of their ability to cleave this type of collagen. Recently, several studies have reported that cancer-derived MMPs can also trigger the proteolytic cleavage of cytokines and their receptors, including tumor necrosis factor (TNF)-receptor (R) (13, 14), interleukin (IL)-6R (14, 15) and IL-2R (16), suggesting that MMPs play an additional role in immune escape of the tumor by disturbing antitumor mechanisms of the host immune system.

We therefore hypothesized that increased levels of MMP-2 (a 72 kDa type IV collagenase, also known as gelatinase A) or MMP-9 (a 92 kDa type IV collagenase, also known as gelatinase B), which are known as key factors of metastasis of OSCC, could reduce the NK cell cytotoxic function against OSCC by proteolytically cleaving or damaging small cytolytic surface molecules on the NK cell. To date our knowledge, no studies have yet explored this hypothesis. We investigated NK cell-mediated cytotoxicity against OSCC cell line by pretreating NK cells with active MMP-2 or MMP-9 *in vitro* and estimated molecular changes in surface moieties of NK cells by flow cytometry.

Materials and Methods

Serum concentrations of MMP-2 and MMP-9 in OSCC patients. In order to estimate the serum level of MMP-2 and MMP-9, sera from 37 patients suffering from OSCC and 20 healthy volunteers were obtained from Oct. 2006 to Apr. 2007 at the Seoul National University Hospital and the Asan Medical Center, Seoul, Korea. The Ethics Committees (Institutional Review Boards) of both institutions approved this study and all patients and volunteers in the study provided written informed consent. The blood samples were drawn before commencement of any treatment such as induction chemotherapy, radiation therapy, or surgery.

The concentrations of MMP-2 and MMP-9 were determined by sandwich enzyme immunoassays. Briefly, these assays employ the quantitative sandwich enzyme immunoassay technique (Quantikine human MMP-2/9 Assay Kit; R&D, Minneapolis, MN, USA). Polyclonal antibodies specific for MMP-2 or MMP-9 are precoated on microplates. Standards and samples are pipetted into the wells, and MMP-2 or MMP-9 is bound by immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for the MMP in question is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of total MMP bound in the initial step. The color development is terminated and the intensity of color measured. The color was measured at 450 nm on a Molecular Devices Thermo Max microplate reader (Molecular Devices, Union City, CA, USA), and calculations were performed using the Soft Max Pro program (Molecular Devices).

Cell culture. To exclude possible variables from other factors between NK cells and MMPs *in vivo*, an *in vitro* study using cell lines and human recombinant (rh)MMPs was designed for further studies. All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). KB cells, a representative OSCC cell line, were cultured in RPMI-1640 medium with 10% (v/v) fetal bovine serum (FBS) (both from GBR, Gaithersburg, MD, USA). The NK-92MI cell line was cultured in alpha medium supplemented with 2 mM L-glutamine, 0.2 mM i-inositol, 20 mM folic acid, 10^{-4} M 2-mercaptoethanol, 12.5% (v/v) FBS, and 12.5% (v/v) horse serum (Myelocult; Stem Cell Technologies, Vancouver, BC, Canada) in the presence of 10-50 units (U) of human IL-2 (Cytolab, Rocky Hill, NJ or R&D, Minneapolis, MN, USA). Culture media were renewed every 2-3 days depending on cell density, and subculture was conducted when confluence was reached.

NK cell-mediated cytotoxicity against OSCC cells. The targets, KB cells, were harvested in the logarithmic phase of growth. Cells (2×10^6) were resuspended in 0.5 ml culture medium. The radiolabel (150 μCi sodium chromate bearing [Cr^{51}]; Amersham Pharmacia Biotech, Little Chalfont, UK) was added and the mixture incubated for 1 h at 37˚C with regular gentle mixing. Following labeling, target cells were thoroughly washed with medium and resuspended in fresh medium to give a final density of 5×10^3 cells/100 μl. Dilutions of washed, cultured NK-92MI cells, after treatments described below, were added to 96-well round-bottomed tissue culture plates, along with 5×10^3 target cells, in a final volume of 200 μl, to provide a range of target/effector ratios. The cells were incubated for 4 h at 37˚C, pelleted by centrifugation at 444 xg for 5 min, and the radioactivity of 100 μl aliquots of supernatant determined using a gamma counter (Packard Cobra, Ramsey, MN, USA). Results are expressed as percentages of specific release (percentage lysis):

$$\% \text{ specific release} = \frac{(\text{experimental release-spontaneous release}) \times 100}{(\text{maximal release-spontaneous release})}$$

Spontaneous release was measured by incubating labeled target cells with 100 μl tissue culture medium, and maximal release by incubating labeled target cells with 100 μl 0.1 M HCl. All tests were performed three or more times. All controls were performed in duplicate, and spontaneous release was always less than 10% of maximal release.

Characterization of NK cells by flow cytometry. Flow cytometric analysis for surface molecules was performed on effector cells (NK92-MI cells) that had been pretreated with active rhMMP-2 or rhMMP-9 (Oncogene, Cambridge, MA, USA) at 100 ng/ml for 48 h. Briefly, following preincubation with or without rhMMP-2 or rhMMP-9, cells were washed twice with phosphate-buffered saline (PBS) pH 7.6, containing 1% (v/v) FBS and 0.1% (w/v) sodium azide (BDH, Poole, UK), pelleted by centrifugation (444 ×g for 3 min), and then incubated with optimal concentrations (predetermined by titration) of the following antibodies: anti-CD94-RPE (anti-IgG1-RPE), anti-NKG2D-APC (anti-IgG1-APC), anti-tumor necrosis factor related apoptosis-inducing ligand (TRAIL) (anti-IgG1-PE), anti-perforin (anti-IgG2B-PE), anti-KIR (anti-IgG2B-PE), anti-FasL (anti-IgG2B-PE) and anti-annexin V-FITC, for 30 min at room temperature. All antibodies were purchased from R&D systems (Minneapolis, MN, USA). The labeled cells were then washed as

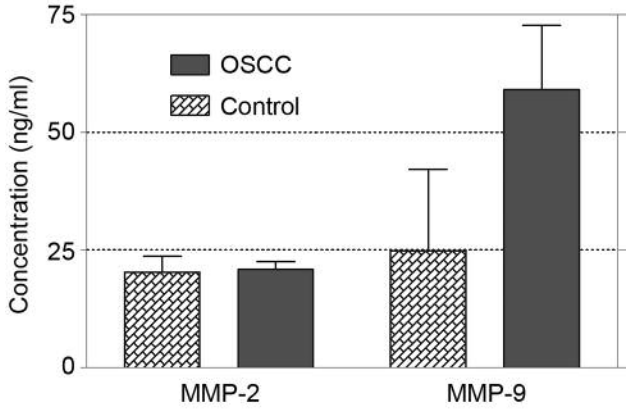

Figure 1. *Serum concentrations of MMP-2 and MMP-9. The serum levels of MMP-2 and MMP-9 were measured by ELISA using microtiter plates coated with recombinant MMP-2 or MMP-9, respectively. The average serum level of MMP-9 was significantly higher in OSCC patients compared to controls (p=0.004), whereas no significant difference was observed for MMP-2.*

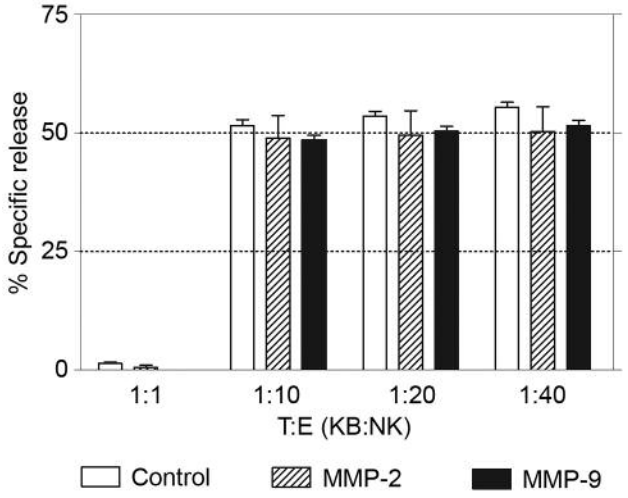

Figure 2. *NK cell-mediated cytotoxicity following pretreatment with either rhMMP-2 or rhMMP-9 at 100 ng/ml for 48 h. Target to effector ratios were 1:1, 1:10, 1:20 and 1:40. Control wells were treated with equivalent amounts of medium alone for 48 h. Percentages of cell lysis were calculated as described in Materials and Methods. The Figure shows averages of three or more experiments.*

before and resuspended in PBS. Data from 10,000 individual cells were acquired using a FACScan and the data were analysed using Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistics. All statistical analyses were performed with the SPSS software system for Windows (version 12.0; SPSS, Chicago, IL, USA). Statistical analyses of the [Cr^{51}] release assay results were made using the Student-Newman-Keuls multiple comparisons test, and a paired *t*-test was employed to analyze the flow cytometric results.

Results

Serum concentrations of MMP-2 and MMP-9 in OSCC patients. The patients ranged in age from 28-87 years (mean age 59.1 years). Using the tumor node metastasis (TNM) classification of the International Union against Cancer, the 37 patients were classified as follows: 5 pT1, 7 pT2, 2 pT3, and 23 pT4 tumors. Pathologically, all the tumors were squamous cell carcinomas. Lymph node metastases were present in 16 out of the 37 patients (43.2%). The mean serum level of MMP-2 in preoperative OSCC patients was 20.83±1.68 ng/ml, whereas that in controls was 20.28±3.39 ng/ml. These numbers showed no significant difference (p=0.744) (Figure 1). The mean serum level of MMP-9 in preoperative OSCC patients was 59.10±13.61 ng/ml, whereas that in controls was significantly lower at 24.83±17.33 ng/ml (p=0.004) (Figure 1). Based on these results, 100 ng/ml of rhMMP-2 or rhMMP-9 was chosen as the concentration of pretreatment of NK cells for further *in vitro* studies.

NK cell-mediated cytotoxicity against OSCC cells. Percentages of specific lysis of KB cells by NK cells pretreated with 100 ng/ml of rhMMP-2 or rhMMP-9 for 48 h, and by untreated control NK cells, were measured using the [Cr^{51}] release assay (Figure 2). Levels of rhMMP-2 and rhMMP-9 used were determined by the serum concentrations of these molecules in OSCC patients. The mean lysis values of KB cells were significantly reduced when NK cells were treated with rhMMP-2 or rhMMP-9, compared to controls at T:E ratios of 1:10, 1:20 and 1:40 (p<0.05).

Flow cytometric analysis of cytolytic moieties on the NK cell surface. Flow cytometric characterization of the NK92MI cell line, with or without exposure to rhMMP-2 or rhMMP-9 at 100 ng/ml for 48 h, was performed with a panel of monoclonal antibodies against potential cytolytic moieties such as perforin, FAS ligand, TRAIL, NK activity stimulatory factor (NKG2D), NK activity inhibitory factor (KIR), CD94, and an apoptosis marker (annexin V). In terms of expression of KIR, significant increases were observed after treatment with rhMMP-2 or rhMMP-9, compared to control cells. With NKG2D, significant decreases were observed, compared to control cells. All data are summarized in Table I.

Discussion

Several studies have shown that the concentrations of plasma or serum MMPs, in particular MMP-2 (17) and MMP-9 (18-21), are increased in patients with cancer. In accordance with previous studies (22, 23), our results with OSCC patients showed that the average level of MMP-9 in the sera of such patients was significantly higher than that of healthy

Table I. *Expression of surface moieties of NK-92MI cells treated with rhMMP-2 or rhMMP-9. Results are representative of three or more experiments (^{NS}, not significant; *p<0.05; **p<0.01).*

Surface markers	Control	rhMMP-2	rhMMP-9
		Positive cell population ± S.E.M. %	
FasL	82.9±4.4	88.2±2.7^{NS}	89.7±2.0^{NS}
Perforin	83.0±4.9	89.7±2.8^{NS}	89.5±2.2^{NS}
TRAIL	72.0±7.6	66.9±8.2^{NS}	67.6±7.5^{NS}
Annexin-V	2.9±0.6	5.5±1.1^{NS}	8.3±2.5^{NS}
CD94	56.6±5.8	52.0±6.1^{NS}	48.9±7.1*
KIR	64.1±3.3	66.8±3.1*	72.1±1.7**
NKG2D	76.3±2.4	68.3±3.1**	62.1±2.2**

volunteers, but the average level of MMP-2 did not differ significantly between OSCC patients and controls. This suggests that an elevated expression of MMP-9 in serum samples may have marker potential in OSCC. A further study, with more OSCC cases, will help to reveal whether circulating MMP-9 could serve as a significant marker of prognosis. The origin of circulating MMP-2 or -9 in cancer patients is still controversial. However, Ruokolainen *et al.* recently showed a significant correlation between MMP-9 immunohistochemical staining levels in tumor tissue and serum levels of MMP-9. This implies that elevated serum MMP-9 levels could at least in part be linked to overproduction of MMP-9 by cancer cells (22).

In our *in vitro* study using the NK-92MI and KB cell lines, we confirmed that the NK cell-mediated cytotoxicity against an OSCC cell line was reduced by pretreatment of the NK cells with active MMP-2 or MMP-9. NK cell-mediated cytotoxicity is mainly controlled by a balance of NK cell inhibitory and activating signals. In humans, the KIRs and the CD94-NKG2 lectin-like receptors are the inhibitory receptors, and NKG2D has been identified as an activating receptor (24, 25). In the present study, expression of KIR and NKG2D showed significant increase and decrease, respectively, in NK cells treated with rhMMP-2 or rhMMP-9, compared to untreated cells. These effects may play a key role in explaining the reduced NK cell cytotoxicity in the present study, and the results support the hypothesis that an immune escape mechanism is mediated by MMPs excreted from tumors. Other surface markers on NK-92MI cells, such as Fas L (CD95), annexin V, perforin, TRAIL, and CD94 also showed some changes in NK cells after MMP treatment, however, these changes were minimal and were not statistically significant (Table I). Notably, CD94/NKG2 is a heterodimer expressed in NK cells, and this receptor may function either as an inhibitor or as an activator depending on which isoform of NKG2 is expressed. Therefore, reduction in CD94 expression by MMP-9 in the present study should be carefully interpreted after further experiments. In addition, the influence of the MMPs on the surface molecules appears to be specific, but further studies will also be required.

In the present study, we measured the serum levels of MMP-2 and MMP-9 in OSCC patients, whereas the effects of MMP-2 and MMP-9 on the NK cell surface marker profile and NK cytotoxicity were measured using the NK-92MI cell line *in vitro*. This *in vitro* experimental design was used mainly to exclude possible variables from other factors such as TGF-β between the MMPs and the NK cells. Although this experimental setting may not reflect the full activity of a complex MMP network *in vivo*, our data suggest that cell death-related molecules on the NK cell surface can be directly influenced by MMPs *in vivo*.

In conclusion, as serum levels of MMP-9 were significantly increased in OSCC patients and the NK cell-mediated cytotoxicity against an OSCC cell line was reduced by MMP-2 and MMP-9, the reduced cytotoxicity may be directly correlated with MMP-induced increases in inhibitory KIR receptors and decreases in activating NKG2D molecules. This study suggests an additional role for MMP-2 and MMP-9 in support of an additional immune escape mechanism of a cancer. Further study will be required to discover the exact interactions between MMPs secreted from cancer cells and host NK cells.

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