Impaired NK Response of Cancer Patients to IFN-α but Not to IL-2: Correlation with Serum Immunosuppressive Acidic Protein (IAP) and Role of Suppressor Macrophage

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Abstract In vitro NK responses of cancer patients (N=21) to rIFN-αA and rIL-2 were examined. The serum concentration of IAP (immunosuppressive acidic protein) was determined in parallel. Five out of seven patients whose serum IAP contents were within the normal range (270 µg/ml to 470 µg/ml), had their NK activities significantly augmented by rIFN-αA and rIL-2. On the other hand, NK cells from ten out of fifteen patients whose serum IAP concentrations were 650 µg/ml or more, were not activated by rIFN-αA. NK cells of these fifteen patients yet were capable of responding to rIL-2. NK cells from cancer patients, however, became responsive to rIFN-αA by either removal of adherent cells or treatment with indomethacin. Therefore, macrophages in PBMC of cancer patients with high serum IAP levels seem to selectively suppress NK response to rIFN-αA by an indomethacin-sensitive mechanisms. It was further shown that PGE2 was not the mediator of this suppression.

Alpha 1-acid glycoprotein (α1-AG) is one of the major acute phase proteins in plasma which increases dramatically when inflammation, pregnancy and cancer occurred (1, 25). We showed that many sera and ascitic fluids obtained from cancer patients contained dramatically increased amounts of α1-AG (18, 23, 24, 29). Furthermore, α1-AG from cancer patients exhibited different compositions of pI subspecies when compared with those derived from healthy or pregnant donors (31). We purified the major subspecies of α1-AG from cancer ascitic fluids to apparent homogeneity (MW: 50 kDa, pI: 3.0). This α1-AG was designated Immunosuppressive Acidic Protein (IAP) (31), since it exhibited immunosuppressive activity in various immunological responses in vitro (2, 31) and in vivo (26), enhanced tumor growth in mice (19), and induced suppressor macrophages (26).

Natural killer (NK) cells are believed to be important effector cells for immunological surveillance against tumor cells and virus-infected cells (8–10).
particular, NK cells have been shown to be involved in prevention of hematogenous tumor dissemination (5, 13, 36). Type I and II interferons (IFNs) and interleukin 2 (IL-2) are well-known as strong activators of NK cells (7, 28, 34), whereas low NK activity has been reported in patients with advanced tumors (27, 30), pregnancy (32, 33), and acquired immunodeficiency syndrome (AIDS) (15).

In the present study, we examined the effect of recombinant human IFNαA (rIFNαA) and rIL-2 on NK activity in peripheral blood mononuclear cells (PBMC) from various cancer patients. Special attention was paid to the relationship between the responsiveness of NK cells to these cytokines and the serum level of immunosuppressive acidic protein (IAP). We found that NK activity in PBMC from cancer patients with high levels of serum IAP responded relatively normally to rIL-2 but very poorly to rIFNαA, and that this selective loss of response to rIFNαA was mediated by co-existing suppressor macrophages.

MATERIALS AND METHODS

Reagents. Recombinant human interferon-αA (rIFN-αA) (specific activity: $5.6 \times 10^7$ units (U)/mg protein) and recombinant human interleukin-2 (rIL-2) ($1 \times 10^6$ U/mg protein) were kindly provided by Nippon Roche Research Center (Kamakura, Japan) and Shionogi Pharmaceutical Co. (Osaka, Japan), respectively. Prostaglandin E2 (PGE2) was provided by Ono Pharmaceutical Co. (Osaka, Japan). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Cell cultures. RPMI-1640 (Gibco, Grand Island, N.Y., U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Microbiological Associates, Walkersville, Md., U.S.A.), 100 U/ml penicillin and 100 μg/ml streptomycin was used as the culture medium throughout the experiments. Peripheral blood mononuclear cells (PBMC) were prepared from healthy adult donors or cancer patients by using Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md., U.S.A.). Nonadherent cells (NACs) were isolated by incubating $3 \times 10^7$ PBMC at 37°C for 1 hr in 10 cm tissue culture dishes (3003, Falcon, Canard, Calif., U.S.A.) that had been precoated with FBS at 4°C overnight. NACs in the culture supernatants were washed three times with the serum-free medium and resuspended in the culture medium. The viability of cell fractions was determined by trypan blue dye exclusion and was >90%. Treatment of PBMC with rIFN-αA or rIL-2 was started 12 hr before NK assay and continued during the assay. Indomethacin was added to the cultures simultaneously with rIFNs or rIL-2.

Assay for NK activity. K-562 cells (16) were labeled with 100 μCi of Na$_2^{51}$ CrO$_4$ (Daiichi Radioisotope Laboratory Co., Tokyo) in 200 μl of 0.85% NaCl solution for 1 hr at 37°C. Cells were extensively washed and cultured in serum-free medium for 1 hr at 37°C. The cells were washed three times and resuspended in the culture medium at $5 \times 10^5$ cells/ml. Preincubation at 37°C for 1 hr in serum-free medium was effective to reduce the spontaneous release of $^{51}$Cr during the assay. Effector cells at varying concentrations in 200 μl were mixed with 20 μl of $^{51}$Cr-labeled target cells in triplicate cultures in U-bottomed microtiter plates (Nunc, Denmark), and
incubated at 37°C for 4 hr in humidified 5% CO₂ air. The plates were centrifuged at 1,200 rpm for 6 min and 100 μl of each supernatant was removed to measure ⁵¹Cr-release. The spontaneous release was determined by incubating target cells without effector cells, and the maximum release was determined by incubating target cells in 3.5 N HCl. The spontaneous release was 8–12% of the maximum release. Specific cytotoxicity and Δ% lysis were calculated as follows:

\[
\% \text{ Specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100;
\]
\[
\Delta \% \text{ Lysis} = \left[\% \text{ specific lysis with treatment}\right] - \left[\% \text{ specific lysis without treatment}\right].
\]

Esterase staining. Esterase staining of monocytes was made on slides prepared by Cytospin 2 centrifuge (Shandon Ltd., England). Nonspecific esterase activity was determined with Esterase Kit (Muto Pure Chemicals Company, Ltd., Tokyo) using α-naphthyl butyrate as substrate (35). Differential counts were performed under a light microscope with a minimum of 500 cells.

Determination of IAP and PGE₂. The serum concentration of IAP was determined with a single radial immunodiffusion method using rabbit anti-IAP serum as described previously (17). The calibration curve with purified IAP was linear between 50 and 1,500 μg/ml. The amount of prostaglandin E₂ in PBMC culture supernatants was determined with a radioimmunoassay kit (New England Nuclear, Boston, Mass., U.S.A.).

Statistical analysis. Data were analyzed for statistical significance by using Student’s t-test.

RESULTS

In Vitro Augmentation of NK Activity by rIFN-αA and rIL-2

We first examined the effect of rIFN-αA or rIL-2 on NK activity in PBMC from healthy adult donors. As shown in Fig. 1, the treatment with rIFN-αA or rIL-2 strongly augmented the cytotoxicity against K-562 cells. The cytotoxicity was linearly correlated with the E/T ratio. The E/T ratio of 5:1 was, therefore, employed in the subsequent experiments. Figure 2 shows the time-course of NK cell activation with different doses of rIFN-αA or rIL-2. Augmentation of NK activity by rIFN-αA reached a plateau level by 6 hr, and 10 U/ml was enough to induce the maximum effect. Augmentation of NK activity by rIL-2 reached a plateau level by 12 hr, and the effect was increasing between 10⁰ to 10⁸ U/ml. Furthermore, rIL-2 generally induced much stronger augmentation than rIFN-αA. After several experiments, 10 U/ml of rIL-2 was chosen as a standard dose for subsequent studies.

Effects of rIFN-αA and rIL-2 on NK Activity in PBMC from Cancer Patients

We next examined the response of NK cells to rIFN-αA and rIL-2 in PBMC obtained from healthy adults and patients with various cancers (N=21). We also
Fig. 1. In vitro activation of NK cells in human PBMC by rIFN-αA or rIL-2. PBMC were incubated with or without 10² U/ml of rIFN-αA or 10 U/ml of rIL-2 for 12 hr, and NK activity against ⁵¹Cr-labeled K-562 target cells was determined at indicated effector-to-target ratios. Medium (●); rIFN-αA (○); rIL-2 (□).

Fig. 2. Kinetics of activation of NK cells in human PBMC by rIFN-αA or rIL-2. PBMC were treated with three different doses of rIFN-αA or rIL-2 for indicated lengths of time and tested for NK cell cytotoxicity in a 4-hr ⁵¹Cr-release assay at 5:1 effector-to-target cell ratio.
measured the serum concentration of IAP, a type of $\alpha_1$-AG, the level of which is known to increase with cancer progression (5, 13, 36). The results are summarized in Fig. 3. The NK activity was strongly augmented by the treatment of PBMC from six healthy donors with rIFN-αA (10^2 U/ml, ○) or rIL-2 (10 U/ml, △). NK cytotoxicity was determined in a 4-hr assay at 5:1 E/T ratio. Closed symbols indicate no statistically significant increases from basal levels. Basal % specific lysis of NK cells in healthy adults was 5.1–24.4% (16.1±8.0%), and those in cancer patients with low serum IAP and high serum IAP were 0–19.6% (9.5±7.8%) and 0–18.6% (5.3±5.7%), respectively [P<0.01, healthy vs high serum IAP-patients].

Fig. 3. The level of increase in % specific lysis of NK cells in PBMC in response to rIFN-αA and rIL-2. PBMC from six healthy donors and twenty-one cancer patients were treated for 12 hr with rIFN-αA (10^2 U/ml, ○) or rIL-2 (10 U/ml, △). NK cytotoxicity was determined in a 4-hr assay at 5:1 E/T ratio. Closed symbols indicate no statistically significant increases from basal levels. Basal % specific lysis of NK cells in healthy adults was 5.1–24.4% (16.1±8.0%), and those in cancer patients with low serum IAP and high serum IAP were 0–19.6% (9.5±7.8%) and 0–18.6% (5.3±5.7%), respectively [P<0.01, healthy vs high serum IAP-patients].
Suppressive Activity of Adherent Cells from Cancer Patients on NK Response to rIFN-αA

Our previous reports showed the increased activity of suppressor macrophages with high serum IAP levels (26, 31). Therefore, we compared the NK response to rIFN-αA of whole PBMC and nonadherent cells (NAC) from six cancer patients who were treated for 12 hr with rIFN-αA (10^2 U/ml), and NK cytotoxicity was determined in a 4-hr assay at 5:1 E/T ratio. Basal % specific lysis of NK cells in cancer patients with 430, 540, 550, 885, 1,000 and 1,010 μg/ml of serum IAP was 15.9, 18.2, 20.4, 8.2, 17.3 and 8.7%, respectively.*No significant increase in NK activity from basal level.

Fig. 4. Restoration of NK response in PBMC from cancer patients to rIFN-αA by depletion of adherent cells. Whole PBMC and nonadherent cells (NAC) from six cancer patients were treated for 12 hr with rIFN-αA (10^2 U/ml), and NK cytotoxicity was determined in a 4-hr assay at 5:1 E/T ratio. Basal % specific lysis of NK cells in cancer patients with 430, 540, 550, 885, 1,000 and 1,010 μg/ml of serum IAP was 15.9, 18.2, 20.4, 8.2, 17.3 and 8.7%, respectively.*No significant increase in NK activity from basal level.

Suppressive Activity of Adherent Cells from Cancer Patients on NK Response to rIFN-αA

Our previous reports showed the increased activity of suppressor macrophages with high serum IAP levels (26, 31). Therefore, we compared the NK response to rIFN-αA of whole PBMC and PBMC depleted of adherent cells. As shown in Fig. 4, NK activities in whole PBMC as well as in the nonadherent cell fraction obtained from three cancer patients whose serum IAP contents were within the normal range (from 430 to 550 μg/ml) were strongly augmented by rIFN-αA. On the other hand, NK activities in PEC were from three cancer patients whose serum IAP levels were high (885–1,010 μg/ml) were not augmented by rIFN-αA. NK cells in the nonadherent cell fraction from the same cancer patients were, however, strongly activated by rIFN-αA. If nonadherent cells were reconstituted with adherent cells as original PBMC, NK response to rIFN-αA was again suppressed (data not shown). On the other hand, NK cells in PBMC and the nonadherent cell fraction from all these cancer patients were significantly activated by rIL-2 (data not shown). Taken together, adherent cells in PBMC from cancer patients with high serum IAP levels seemed to selectively suppress NK activation by rIFN-αA.
Enumeration of Monocytes in PBMC from Cancer Patients

The suppressive effect of adherent cells from cancer patients with high serum IAP levels on NK response to rIFN-αA might be due to their increase in PBMC. Therefore, we determined the numbers of monocytes in PBMC by nonspecific esterase staining. The results are summarized in Table 1. Six healthy adults whose serum IAP contents ranged from 230 µg/ml to 420 µg/ml showed 19.1 ± 2.6% esterase-positive cells in PBMC. The percentage of esterase-positive cells in PBMC from ten cancer patients whose serum IAP contents ranged from 230 µg/ml to 570 µg/ml (similar to the normal level) was 20.7 ± 3.8%. The percentage of esterase-positive cells in PBMC of the third group, five cancer patients with high serum IAP levels (959 ± 119 µg/ml), was 20.0 ± 2.1%. Thus there was no significant difference in the number of esterase-positive cells in PBMC among these three groups. These results suggest that the suppressive effect of monocytes in PBMC from cancer patients with high serum IAP levels was probably due to altered functional properties but not due to a simple increase in their number.

Effect of Indomethacin on NK Response to rIFN-αA

Previous studies have demonstrated that suppressor macrophages inhibit immune responses mainly by releasing prostaglandins (4, 6). Since indomethacin (1 µM/ml) can inhibit the production of prostaglandins (20), we examined the effect of indomethacin on NK response to rIFN-αA in PBMC from cancer patients. As shown in Fig. 5, indomethacin restored the NK responsiveness to rIFN-αA of PBMC from cancer patients whose serum IAP levels were 640–1,540 µg/ml.

Suppressor Macrophages and PGE2 Production

The effect of indomethacin prompted us to determine the PGE2 production in PBMC from cancer patients. The amount of PGE2 produced in culture supernatants of PBMC from three cancer patients did not correlate with either low NK response to rIFN-αA or high serum IAP levels. The treatment of PBMC with rIFN-αA did not increase the production of PGE2 (Table 2). In fact, the treatment consistently reduced the production of PGE2. Even though suppressor macrophages

<table>
<thead>
<tr>
<th>Donor</th>
<th>Serum IAP (µg/ml)</th>
<th>% Esterase-positive cells&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy adults (n=6)</td>
<td>311 ± 62&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>19.1 ± 2.6</td>
</tr>
<tr>
<td>Cancer patients</td>
<td></td>
<td></td>
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<tr>
<td>Low serum IAP (n=10)</td>
<td>384 ± 121</td>
<td>20.7 ± 3.8</td>
</tr>
<tr>
<td>High serum IAP (n=5)</td>
<td>959 ± 119</td>
<td>20.0 ± 2.1</td>
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<sup>a</sup) Nonspecific esterase positive cells were determined on Cytospin slides with α-naphthyl butyrate as substrate. Differential counts were performed on a minimum of 500 cells.

<sup>b</sup) Mean ± S.D.
in PBMC from cancer patients seem to inhibit NK response to rIFN-αA by an indomethacin-sensitive mechanism, the inhibition is not due to an increase in production of PGE₂.

**DISCUSSION**

The NK activity of 6 healthy adults and 21 cancer patients was compared (Fig. 3). It has been reported that NK cell activity markedly declines in humans (27, 30) and animals (22, 37) with advanced cancers. In the present study, we did
not observe any consistent decrease in NK activity in cancer patients, even though we noticed that the basal levels of NK cell activity in cancer patients varied with a wide range of deviation. NK cell activity of healthy adults was strongly augmented by rIFN-αA or rIL-2. NK cell activity of cancer patients whose serum IAP levels were at the normal range was also augmented by those cytokines. In contrast, NK cells from cancer patients with high serum IAP levels (>650 μg/ml) could be activated only by rIL-2 but not by rIFN-αA, even though the number of cells with Leu-11+ NK marker (14) were quite similar among those three groups of donors (data not shown). Poor responses of NK cells in PBMC from cancer patients with high serum IAP levels were not restricted to type I IFN. When we tested the effect of rIFN-γ (10² U/ml) on NK cell activity in PBMC, no augmentation was shown for two cancer patients whose serum IAP levels were 690 and 1,310 μg/ml, even though NK cells from six healthy adults were significantly activated by rIFN-γ (data not shown).

Regulation of NK cell activity by monocytes has been shown by Bloom and Babbitt (3). We found that there was no difference in the number of monocytes (esterase-positive cells) in PBMC from healthy adults and cancer patients (Table 1). However, after removing adherent cells (90–95% monocytes) from PBMC of cancer patients with high IAP serum levels, NK cells became responsive to rIFN-αA (Fig. 4). When adherent cells were added back to the nonadherent cells, the activation of NK cells by rIFN-αA was again suppressed (data not shown). These results strongly suggest that the impaired response of NK cells in cancer patients with high serum IAP levels to rIFN-αA is due to suppressor monocytes.

Monocytes in PBMC from cancer patients with high serum IAP levels have been shown to suppress various immune responses (31). Since the addition of indomethacin restored the NK response of PBMC from cancer patients with high serum IAP levels to rIFN-αA (Fig. 5), we initially presumed that the increased PGE₂ production from monocytes might be responsible for their suppressive activity. However, the amounts of PGE₂ produced in culture supernatants of PBMC from three cancer patients were rather reduced by rIFN-αA (Table 2). These results indicate that the selective suppression of NK response to rIFN-αA in cancer patients is not due to PGE₂ production from suppressor monocytes upon stimulation with rIFN-αA.

The question still remains as to why patients with a high serum IAP level had suppressive monocytes in response to rIFN-αA but not to rIL-2. Though IFN-γ induces surface expression of receptors for IL-2 with normal human monocytes, freshly isolated monocytes obtained from PBMC are known to be originally IL-2 receptor negative (11, 12). On the other hand, monocytes in PBMC of healthy adults are known to have receptors for IFNs, thus resulting in the modification of several immunological functions of monocytes after the treatment with IFNs (21). Monocytes in PBMC from cancer patients with high serum IAP levels are already known to show the ability to suppress immune responses in various ways (31). In our understanding, the failure of IFNs to abolish monocyte suppressive activity in these patients was counteracted by the addition of indomethacin, which did abolish
monocyte suppressive activity, probably through the monocyte IFN receptor sites. Further studies are required to elucidate the mechanism of action of suppressor macrophages in cancer patients.

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REFERENCES


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