Susceptibility of NB-I Neuroblastoma Cells to Tumoricidal Activity of Monocytes Activated by γ-Interferon

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The purpose of this study was to examine the susceptibility of NB-I human neuroblastoma cells to direct cellular cytotoxicity mediated by peripheral blood monocytes from pediatric cancer patients receiving chemotherapy. Nonactivated monocytes from patients showed spontaneous cytotoxicity to NB-I neuroblastoma cells (37 ± 18%) but only marginal cytotoxicity to A375 melanoma cells (21 ± 14%) at the effector:target cell ratio of 20:1. This spontaneous cytotoxicity to NB-I cells was observed only after > 24 h of cocultivation and was proportional to the effector:target cell ratio. Activation of monocytes by recombinant human interferon γ (rIFN) (1 × 10⁴ U/ml) consistently and strongly enhanced their tumoricidal activity to NB-I cells (87 ± 6%) and this tumoricidal activity was even superior to that observed against A375 cells, which are known to be extremely sensitive to lysis by activated monocytes. In contrast, activation of monocytes by lipopolysaccharide (LPS, 1 μg/ml) had no effect on monocyte-mediated lysis of NB-I cells, while A375 cells were equally lysed by rIFN- and LPS-activated monocytes, thus suggesting that different mechanisms are involved in the monocyte-mediated lysis of A375 melanoma and NB-I neuroblastoma cells. Susceptibility of the neuroblastoma cell line to monocyte-mediated cytotoxicity has not been reported so far and our results may have some clinical implication if this observation can be extended to other neuroblastoma cell lines as well.

Key words: Neuroblastoma — Monocyte — Tumor cytotoxicity — Interferon γ

Clinical application of biological response modifiers such as γ interferon (rIFN) has been tried in the treatment of neuroblastoma (NBL), which is the most common solid abdominal tumor in childhood. This is based on the in vitro observation that NBL cells are sensitive to lysis by natural killer cells and that rIFN can enhance this tumoricidal activity. Another rationale for using rIFN is that it may enhance T-cell-mediated cytotoxicity against NBL cells by induction of class I antigenic expression on their surface. In this report we demonstrate that an NBL cell line, NB-I, is lysed via direct cellular cytotoxicity mediated by activated monocytes; this constitutes another important effector mechanism of natural immunity against tumor development. This tumoricidal property is strongly augmented by activation of monocytes by rIFN but not by lipopolysaccharide (LPS). It is of particular interest that activated monocytes from pediatric cancer patients receiving intensive chemotherapy also exhibit strong lytic activity against NBL cells.

MATERIALS AND METHODS

Reagents and chemicals RPMI 1640, Eagle's minimum essential medium and Hank's balanced salt solution (HBSS) were purchased from Sigma Chemical Co., St Louis, MO. All reagents were free of endotoxins as determined by the Limulus amebocyte lysate assay. Bacterial LPS (E. coli 055:B5) was purchased from Sigma Chemical Co. rIFN was provided by Shionogi Pharmaceutical Co., Osaka. Cell cultures The NB-I line, derived from a human neuroblastoma, was obtained from the Japanese Cancer Research Resources Bank (JCRB). The A375 line, derived from a human melanoma, was kindly provided by Dr. S. Sone, Tokushima University, Japan. Both cell lines were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine (CMEM) and their tumorigenic potential was verified by their ability to cause tumors in nude mice. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Patients Six patients were studied for monocyte-mediated tumoricidal activity against NBL cells. Four patients with acute lymphoblastic leukemia (ALL) were in remission and were receiving the intensive multidrug regimen of the Japanese Children's Cancer Study Group and one neuroblastoma patient was receiving the adjuvant chemotherapy protocol of the same group. One patient with rhabdomyosarcoma was treated in accordance to the protocol of the Intergroup Rhabdomyosarcoma Study (each protocol available on request). Blood sampling was done before administration of the scheduled chemotherapy when the patients had enough WBC counts (> 5,000/mm³). For control studies, blood specimens were obtained from 8 normal donors.
Isolation of monocytes  Mononuclear leukocytes (MNL) were isolated from heparinized peripheral blood on Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), washed twice in HBSS, and then suspended in RPMI 1640 containing 5% human AB serum. Cell number was determined on a hemocytometer and differential cell counts were performed with either May-Giemsa or peroxidase stain of the cytospin slides, and the cell suspension was adjusted to contain 1 × 10^6 monocytes/ml. Into each well of a 96-well flat-bottomed Microtest II plate (Falcon, Oxnard, CA), 2 × 10^5 monocytes were added and allowed to adhere for 1 h at 37°C. Nonadherent cells were removed by 3 washes with HBSS. The purity of the adherent monocyte monolayers obtained by this method is >97%. Purified monocytes were then incubated at 37°C for 18-24 h with 0.2 ml of control medium, medium containing rIFN (1 × 10^4 U/ml), or medium containing LPS (1 μg/ml). After this incubation period, the adherent monocyte cultures were washed twice with HBSS, and [1^25I]iododeoxyuridine (125I­UdR)-labeled target cells were added as described below.

Assay of monocyte-mediated cytotoxicity against NBL cells  Monocyte-mediated cytotoxicity was assessed by the method described by Kleinerman et al.9) Target cells were incubated for 24 h in CMEM containing 125I­UdR (0.3 μCi/ml: specific activity 185 GBq/mg; Amersham, Chicago, IL). Target cells were washed twice and harvested by a 1-min trypsinization with 0.25% trypsin + 0.02% EDTA (Sigma Chemical Co.). Labeled target cells were resuspended in CMEM and added to the culture wells with the monocyte cultures at the indicated effector:target cell (E/T) ratio. Radiolabeled target cells were also plated alone as an additional control group. After 24 h, the cultures were washed to remove the nonadherent target cells, refed with fresh medium, and cultured for an additional 0-48 h. The cultures were then washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter. The cytotoxic activity of the monocytes was calculated as follows:

Endogenous (spontaneous) % cytotoxicity
= (A − B)/A × 100

where A is cpm in target cells cultured alone, and B is cpm in target cells cultured with untreated monocytes.

% cytotoxicity of activated monocytes
= (A − C)/A × 100

where C is cpm in target cells cultured with treated monocytes. Values of < 0% cytotoxicity were expressed simply as 0% cytotoxicity.

Microscopic examination  The interaction between monocytes and NBL cells was observed microscopically.

RESULTS

Time course study  The radioisotope-labeled NB-I cells were added to the monocyte cultures from normal controls and the cytotoxicity was assessed by measuring the release of radioactivity 24, 48, and 72 h later (Fig. 1).

Monocytes (2.8 × 10^6) were plated on coverslips in 24-well tissue-culture plates and cultured for 18 h in the presence or absence of rIFN or LPS. NBL cells were added 24 h later so that the effector:target ratio was 20:1, and the cells were cocultured for 72 h. Cells grown on coverslips were washed twice with HBSS and stained with Wright-Giemsa solution.

Statistical analysis  The statistical significance of differences was evaluated by the use of Student's t test.

FIG. 1. Time course study on tumoricidal activity of monocytes against NB-I neuroblastoma cells. [125I]UdR-labeled NB-I cells were added to cultures of nonactivated or rIFN-activated monocytes at the indicated effector:target cell ratio and cocultivated. Cultures were terminated at 24-72 h and assayed for cytotoxicity by counting the radioactivity of the residual target cells.

○ — ○ nonactivated monocytes E/T = 20/1.
○ — ○ nonactivated monocytes E/T = 10/1.
• — • rIFN-activated monocytes E/T = 20/1.
• — • rIFN-activated monocytes E/T = 10/1.
NB-I cells were not lysed after 24 h of cocultivation with nonactivated monocytes at the E/T ratio of either 10/1 or 20/1. Significant tumoricidal activity (> 15%) was observed when the cocultivation time exceeded 24 h, and reached the maximum at 72 h.

**Effect of the effector: target cell ratio on tumor cytotoxicity** Cytotoxic activity of monocytes was proportional to the E/T ratio (Fig. 2). The levels of cytotoxic activity of both nonactivated and activated monocytes exceeded 60%, which is considered to be the maximum cytotoxic level of this assay. Considering these results, the cocultivation time of 72 h and the E/T ratio of 20/1 were considered to be optimal for this assay and were therefore chosen for the later studies.

**Susceptibility to lysis by monocytes from patients** Tumoricidal activity of monocytes from patients was compared to that of normal individuals in the parallel setting. In addition, A375 melanoma cells, which are extremely sensitive to monocyte-mediated tumor cytotoxicity, were used as a positive control. Monocytes from normal individuals showed no spontaneous cytotoxicity to A375 cells. Activation of these monocytes either by rIFN or LPS rendered them cytotoxic to A375 cells (Table I). Monocytes from the patients showed spontaneous cytotoxicity to this target, albeit at the marginal level (21.5%), and their tumoricidal activity was augmented to the same level as that of normal individuals by these activating agents (Table I). In contrast, significant spontaneous cytotoxicity was observed against NB-I cells by monocytes from both the normal individuals and cancer patients. This tumoricidal activity was strongly enhanced by activation of monocytes by rIFN. Activation of monocytes by LPS, however, showed no enhancement and rather reduced their tumoricidal activity. These results are summarized in the table.

**Microscopic examination** There was a marked reduction of the number of NB-I cells when they were cocultivated with monocytes activated by rIFN. This was not observed when they were cocultivated with control monocytes or monocytes activated by LPS. At higher magnification, monocytes were seen to be frequently in contact with the target cells. Some of these monocytes were located very close to the nuclei of the NB-I cells and they produced a clear zone around them in the cytoplasm of NB-I cells (Fig. 3). This was observed only in the interaction between the NB-I cells and the monocytes activated by rIFN.

### Table I. In vitro Lysis of A375 Melanoma and NB-I Neuroblastoma Cells by Human Blood Monocytes from Normal Controls and Patients

| Target            | Monocytes | % Cytotoxicity of non-activated monocytes | % Cytotoxicity of rIFN-activated monocytes | % Cytotoxicity of LPS-activated monocytes |
|-------------------|-----------|------------------------------------------|-------------------------------------------|-----------------------------------------|
| A375 melanoma     | Control   | 10±8                                     | 50±8 (40)†                                | 43±18 (33)                              |
|                   | Patient   | 22±14                                    | 43±11 (21)                                | 46±10 (24)                              |
| NB-I neuroblastoma| Control   | 54±27                                    | 91±5 (37)                                 | 46±30                                   |
|                   | Patient   | 37±18                                    | 87±6 (50)                                 | 15±19                                   |

† Numbers in parentheses are the percent cytotoxicity generated by activated monocytes (P<0.05).
DISCUSSION

rIFN has been administered to patients with various types of refractory cancer with some positive results.\textsuperscript{1,10-13} Antitumor activities of rIFN are attributed to either the direct antiproliferative effects or the stimulation of host defense mechanisms. It is likely, however, that the mechanisms responsible for tumor cell lysis are different according to each target cell type. Although the effects of rIFN on the biological activities of natural killer cells and cytotoxic T-cells have been extensively studied in the treatment of NBL,\textsuperscript{1,4} little emphasis has been given to the effects on antitumor action of human blood monocytes against NBL cells. In this study, we demonstrated that an NBL cell line, NB-I, is highly susceptible to lysis by monocytes activated by rIFN as judged from both radioisotope release assay and morphological observation. Although we tried other neuroblastoma cell lines such as IMR-32 and GOTO, they were not appropriate for our assay system because of a tendency to aggregate (IMR-32) or poor labeling (GOTO). Therefore, the generality of susceptibility of neuroblastoma cells to monocyte-mediated cellular cytotoxicity remains to be confirmed by using other cell lines or other assay systems.

The assay system we employed was the same as the one that Kleinerman \textit{et al.} have been using. They have been studying extensively the tumoricidal properties of the monocytes by this method and demonstrated that the effector cells in this assay system are the monocytes and not natural killer cells that might be contaminating the adherent cell preparations to a minor extent.\textsuperscript{14}

Since monocyte cultures were washed thoroughly before the addition of the target cells, it is unlikely that NB-I cells were lysed via the direct antiproliferative effects of residual rIFN. In fact, there was no difference in the radioactivity when the isotope-labeled NB-I cells were harvested after cultivation in the presence or absence of rIFN in the culture medium (data not shown). We used the A375 line in the parallel setting as a positive control in our study. This cell line is extremely sensitive to monocyte tumoricidal activity, which has recently been shown to correspond to interleukin-1 and tumor necrosis factor secreted by the activated monocytes.\textsuperscript{15-17} In fact, very small spontaneous cytotoxicity to A375 cells was observed by the non-activated monocytes, while they were equally well lysed by both rIFN- and LPS-activated monocytes. In contrast, spontaneous cytotoxicity to NB-I cells was observed in most of the patients and normal individuals in our study. Interestingly, this tumoricidal activity to NB-I was enhanced only by rIFN and not by LPS. We do not have any explanation for these paradoxical results. However, it is reasonable to assume that the principal mediators responsible for lysis of NB-I and A375 cells might be different, since these experiments were performed in the parallel setting using identical effector cells.

Our observation that NBL cell line NB-I is lysed by monocytes activated by rIFN may have some clinical implication, since rIFN has already been used for the treatment of neuroblastoma. It has been administered in...
T-cell-mediated cytotoxicity toward NBL cells. The hope of enhancing either the natural killer activity or T-cell-mediated cytotoxicity toward NBL cells. Susceptibility of the NBL cell line to monocyte-mediated cellular killing in the rIFN treatment of neuroblastoma. The involvement of this effector mechanism should be studied further. Cytotoxicity has not been reported so far and possible modulation by interferon should be examined.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Aichi Cancer Research Foundation.

(Received March 22, 1990/Accepted July 9, 1990)

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