Contribution of Inflammatory Cytokine Release to Activation of Resident Peritoneal Macrophages after \textit{in vivo} Low-dose \(\gamma\) -irradiation

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The activation mechanism of resident peritoneal macrophages by \textit{in vivo} \(\gamma\)-irradiation was investigated. The function of macrophages as accessory cells in concanavalin A-induced proliferation of spleno-lymphocytes (accessory function) was enhanced 4 h after a low-dose irradiation (4 cGy) \textit{in vivo}, but not \textit{in vitro}, indicating that low-dose irradiation acts indirectly on the activation of macrophages. Because we expected that macrophages were activated by the recognition of substances damaged by \textit{in vivo} irradiation, we co-cultured macrophages with oxidized erythrocyte-ghosts. No change was found in their accessory function. The production of inflammatory cytokines, interleukin-1\(\beta\) (IL-1\(\beta\)) and interferon-\(\gamma\) (IFN-\(\gamma\)), in the supernatant of cocultures of spleno-lymphocytes and macrophages was determined by an ELISA. Production of both increased in the presence of \textit{in vivo} irradiated macrophages. Furthermore, IL-1\(\beta\) production from \textit{in vivo}-irradiated macrophages treated with recombinant IFN-\(\gamma\) also was enhanced. The mRNA expression of the cytokines released from macrophages and lymphocytes was determined by RT-PCR. Increases in IL-1\(\beta\) mRNA expression were found in both \textit{in vivo}- and \textit{in vitro}-irradiated macrophages. \textit{In vivo} irradiation also enhanced the expression of IFN-\(\gamma\) mRNA in lymphocytes, whereas there was no change after \textit{in vitro} irradiation. On the basis of these observations, we propose that the activation of macrophages is caused by interaction with neighboring cells, such as lymphocytes, and by paracrine induction of certain cytokines which is initiated by the small amount of IL-1\(\beta\) released by irradiated macrophages.

INTRODUCTION

We elsewhere reported that low-dose (2 cGy) \textit{in vivo} irradiation enhanced the concanavalin A (Con A)-induced proliferation of splenocytes, which was caused not by activation of spleno-lymphocytes, but by activation of macrophages, accessory cells\(^{1,2}\). Macrophages are

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fairly radio-resistant relative to other eukaryotic cells. For example, the tumoricidal activity of alveolar macrophages obtained from C57BL/6 and BALB/c mice is reported not to be significantly affected by $\gamma$-irradiation at 50 or 100 Gy$^3$, whereas that of exudate peritoneal macrophages from BALB/c mice is enhanced by $\gamma$-irradiation at 100 Gy$^4$. We also reported that high-dose (6 Gy) in vitro-irradiated macrophages showed enhanced nitric oxide or superoxide production, and tumoricidal activity$^5$. No changes, however, were found in accessory function or nitric oxide production 4 h after in vitro irradiation at 4–200 cGy$^{1,2}$. We therefore became interested in the mechanism of activation of macrophages induced by very low-dose in vivo irradiation.

Macrophages are unique in their responses to external stimuli$^6$: 1) they recognize, phagocytose and lyse foreign particulate materials, as well as damaged or dead cells; 2) they interact with and internalize various other molecules, such as proteins and polysaccharides; 3) parts of the protein and peptide molecules salvaged from their extensive lysis are presented to lymphocytes in a biochemically modified form; 4) they are responsible for the production of inflammatory and growth regulatory factors, such as interleukin-1 (IL-1) and arachidonate derivatives; and 5) they have surface receptors for lymphokines, the regulatory proteins released by lymphocytes. The activation of macrophages induced by low-dose in vivo irradiation therefore may be caused by the interaction with lymphocytes and released lymphokines, or by the recognition of some other molecules or cells damaged by irradiation.

We examined the effects of in vivo and in vitro irradiation on macrophage accessory function and changes in function in macrophages that had been treated with oxidized or irradiated erythrocyte-ghosts. Because ionizing radiation is associated with increased expression of inflammatory cytokines in various cell types$^7-^{11}$, we also determined IL-1$\beta$ mRNA expression in macrophages and IFN-$\gamma$ mRNA expression in lymphocytes, as well as the production of these proteins in cocultures of spleno-lymphocytes and macrophages, based on the supposition that macrophages and lymphocytes may mutually modify their behavior through the release of bioactive molecules after low-dose in vivo irradiation.

MATERIALS AND METHODS

Materials

Male C57BL/6 mice, 7 weeks of age, were obtained from Japan SLC, Inc., Japan. Eagle’s minimum essential medium (MEM) and RPMI1640 medium were products of Nissui Pharmaceutical Co., Japan, and fetal bovine serum (FBS) was from JRH Biosciences, USA. Con A and recombinant mouse IFN-$\gamma$ respectively were purchased from Honen Co., Japan and Genzyme Co. USA. $^3$H-Thymidine (555 GBq/mmol) was obtained from New England Nuclear Research Products, USA.

For the PCR assay, QuickPrep Micro mRNA purification and GeneAmp RNA-PCR reaction kits respectively were purchased from Pharmacia Biotec, USA and Perkin Elmer, USA.
γ-Irradiation

Mice, obtained cells (macrophages, spleno-lymphocytes) and erythrocyte-ghosts were irradiated with γ-rays using an Irradiation Exposure System (Pony Atomic Industry, PS-600SB, 137Cs: 22.2 TBq). Doses were 4 or 400 cGy and the respective rates 0.2 or 20 cGy/min. In all experiments, the controls were sham-irradiated.

Measurement of the accessory function of macrophages

Resident peritoneal macrophages and spleno-lymphocytes were prepared as described previously 1). Briefly, macrophages were obtained by washing out the peritoneal cavities of C57BL/6 mice and removing nonadherent cells. Spleno-lymphocytes were prepared by passing mouse splenocytes through a Sephadex G-10 column. Mixtures of 100 μl of spleno-lymphocytes (5 ¥ 10^6 cells/ml) and 50 μl of macrophages (1 ¥ 10^5 cells/ml) were cultured at 37°C for 48 h in the presence of 50 μl of Con A (8 μg/ml). 3H-Thymidine was added 15 h before cell harvest, and the radioactivity of the collected cells was measured with a liquid scintillation counter (LSC-3100, Aloka, Japan). In the in vivo experiments, macrophages were obtained 1 or 4 h after whole-body irradiation. In the in vitro experiments, macrophages were cultured at 37°C in an incubator for 1 or 4 h in 5% CO_2 after irradiation.

Preparation of oxidized erythrocyte-ghosts

Hemoglobin-depleted erythrocyte-ghosts were prepared as follows. Fresh blood taken from the same mouse from which macrophages had been obtained was centrifuged (1,500 ¥ g, 5 min, 25°C) three times with phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) (PBS (–)). The erythrocytes were lysed in 30 volumes of hypotonic hemolysis buffer (10 mM Tris-HCl (pH 7.4)), centrifuged (34,000 ¥ g, 15 min, 4°C), then resuspended in the same buffer. This procedure was repeated three or four times until the ghosts showed grayish-white, after which they were treated with t-butyl hydroperoxide (5 mM) for 30 min or irradiated at 4 or 400 cGy in RPMI1640 medium at 37°C.

After centrifugation (34,000 ¥ g, 15 min, 4°C), the ghosts were resuspended and co-cultured with macrophages for 1 h at 37°C. After two washings of the macrophages, their accessory functions were examined as described above.

Determination of IL-1β and IFN-γ in the culture supernatants

Mixtures of unirradiated spleno-lymphocytes (5 ¥ 10^5 cells/100 μl) and macrophages (5 ¥ 10^3 cells/50 μl) obtained 4 h after in vivo γ-irradiation were cultured at 37°C for 48 h in the presence of 50 μl of Con A (8 μg/ml). In an another experiment, macrophages (3 ¥ 10^4 cells/100 μl) were cultured for 48 h in the presence of 100 μl of recombinant IFN-γ (400 U/ml). The supernatants (50 or 100 μl) were used in the quantitative determinations of IL-1β and IFN-γ by an ELISA kit (Biosource International Inc., USA).

Determination of IL-1β mRNA in macrophages and IFN-γ mRNA in lymphocytes by RT-PCR

In the in vivo irradiation experiment, spleno-lymphocytes and macrophages were prepared 4 h after whole-body irradiation. In the in vitro experiment, they were allowed to
stand in 5% CO\textsubscript{2} at 37°C for 24 h after preparation then irradiated, after which they were kept in a CO\textsubscript{2} incubator for 1 h then washed three times with PBS (–). Poly (A)	extsuperscript{+} mRNA was isolated using a QuickPrep Micro mRNA purification kit. The RT-PCR was done with a GeneAmp RNA-PCR reaction kit according to the manufacturer’s protocol. The extracted mRNA (100 ng) was incubated for 10 min at room temperature in amplification buffer (10 mM Tris-HCl, pH 8.3, 5 mM MgCl\textsubscript{2} and 4 mM deoxynucleotide triphosphate) containing 1 U/μl RNase inhibitor, 2.5 μM oligo dT primer and 2.5 U/μl reverse transcriptase at the final volume of 20 μl. The samples then were placed in a thermal cycler, heated to 42°C for 15 min, then to 99°C for 5 min, and lastly cooled to 4°C. Taq polymerase (2.5 U) and 10 ng of each oligonucleotide primer (IL-1β, sense: 5’-GCTACCTGTGTCTTTCCCCGTG-3’, antisense: 5’-GTCTAATGGGAACGCACACACC-3’, IFN-γ, sense: 5’-CCCTCATGCGTTGCTTGCTG-3’, antisense: 5’T-TCCTGGCTGGACC-3’, β-actin, sense: 5’-TGTAATGTTGAGACCCGAG-3’, antisense: 5’-CTGCGGATGTCAGAA-3’, final volume of 100 μl, were added and denatured for 105 sec at 95°C. The PCR was performed for 25–30 cycles of denaturation (95°C, 15 sec), annealing (59°C, 30 sec) and extension (72°C, 15 sec). On completion of the last cycle, the samples were kept at 4°C.

After PCR amplification, the samples were electrophoresed in 2.5% agarose gels containing 0.1 μg/ml ethidium bromide and made visible with a UV illuminator. The ratios of the expression of the cytokines to β-actin were determined with a FluorImager (Molecular Dynamics, Inc., Japan).

Statistics

The experiments were repeated two or three times. Values given are the means of measurements (n = 4 ~ 10). Student’s t-test was used to test the significance between groups.

RESULTS AND DISCUSSION

Effects of in vivo and in vitro low-dose irradiation on the accessory function of macrophages

Figure 1 shows the time course of the effects of in vivo and in vitro irradiation on the function of macrophages as accessory cells in Con A-induced proliferation of spleno-lymphocytes. Data on the in vivo experiment were cited from our previous paper\textsuperscript{1}. In the absence of macrophages, spleno-lymphocytes had a low level of \textsuperscript{3}H-thymidine incorporation. The addition of macrophages induced proliferation which increased with time after the in vivo irradiation, whereas no enhancement occurred in vitro. This finding that enhancement was induced only by in vivo irradiation suggests that the activation of macrophages was induced indirectly; that is, other cells and their products released after in vivo irradiation participated in the activation. Why \textsuperscript{3}H-thymidine incorporation in the in vitro experiment was approximately double that in the in vivo experiment could not, however, be explained.
Effects of oxidized erythrocyte-ghosts on the accessory function of macrophages

Macrophages recognize, phagocytose and lyse foreign or effete particulate materials such as microorganisms, senescent erythrocytes\(^{12}\) and apoptotic cells\(^{13}\). In this process, parts of the protein and peptide molecules salvaged from the extensive lysis serve as substrates for recognition by T lymphocytes\(^6\). This reaction leads to the activation of lymphocytes. Ionizing radiation is considered to induce apoptosis or a cellular transformation, such as lipid peroxidation, through the production of active oxygen species e.g. OH\(^-\) etc. The erythrocytes modified by oxidizing agents are reported to cause lipid peroxidation and to be recognized by macrophages\(^{12}\). We selected oxidized or irradiated erythrocyte-ghosts as the substances recognized and phagocytosed by macrophages and examined their effects on macrophage activation. Erythrocytes were obtained from the same mouse as were the macrophages, because macrophages recognized and phagocytosed cells from other individual animals. Macrophages did not recognize non-treated erythrocytes, whereas they phagocytosed many of those oxidized by \(t\)-butyl hydroperoxide (data not shown). The accessory function in the Con A-induced proliferation of spleno-lymphocytes did not, however, change in macrophages treated with irradiated erythrocyte-ghosts (4, 400 cGy), not even in macrophages which phagocytosed the erythrocyte-ghosts oxidized by \(t\)-butyl hydroperoxide (Fig. 2). These findings indicate that no change in the accessory function occurred even in macrophages which recognized cellular materials transformed by irradiation and the attendant production of active oxygen.
Interaction of macrophages and lymphocytes through inflammatory cytokine release

Interleukin 1 (IL-1), a cytokine with a broad range of biological activity\textsuperscript{14,15}, acts predominantly as an intercellular mediator. Activated macrophages and other antigen-presenting cells are responsible for the production of IL-1, which leads to B cell proliferation\textsuperscript{16,17} and T cell activation accompanied by increased production of lymphokines (IL-2, IFN-\(\gamma\) etc.)\textsuperscript{15,17,18}. Furthermore, the IFN-\(\gamma\) produced activates macrophages\textsuperscript{19}. Ionizing radiation and oxidative injury are reported to enhance the release of IL-1 from macrophages and monocytes\textsuperscript{8–10,20,21}. Therefore, we suppose that low-dose irradiation induced IL-1 release from macrophages, which then led to an increase in lymphocyte proliferation.

Figure 3A shows the IL-1\(\beta\) and IFN-\(\gamma\) productions in the culture supernatant after spleno-lymphocytes were co-cultured with macrophages in the presence of Con A. In the absence of macrophages, neither IL-1\(\beta\) nor IFN-\(\gamma\) production occurred, whereas the addition of macrophages induced both productions which were enhanced even more by the \textit{in vivo}
irradiation to macrophages. Furthermore, in vivo-irradiated macrophages enhanced IL-1β production by treatment with recombinant IFN-γ (Fig. 3B). These findings suggest that cytokines participate in the activation of macrophages. Figure 4 shows the expression of IL-1β mRNA in peritoneal macrophages and IFN-γ mRNA in spleno-lymphocytes 4 h after in vivo irradiation. PCR cycles were determined from the points where the reactions proceeded exponentially. The expression levels of both IL-1β and IFN-γ mRNA, which were detected constitutively, were increased by low-dose in vivo irradiation. In the in vitro experiment (Fig. 5), cells obtained from mice were kept for 24 h in 5% CO₂ in an incubator to minimize constitutive cytokine expression. Low-dose in vitro irradiation enhanced IL-1β mRNA expression in the macrophages, whereas IFN-γ mRNA expression in the lymphocytes did not change. Ishihara et al. earlier reported an immediate-early and transient increase of IL-1β mRNA in spleen cells after in vitro high-dose irradiation, in contrast to an immediate-early and continuous, not transient, increase after in vivo irradiation. These findings suggest regulatory mechanisms mediated by cell-cell interaction in vivo.

On the basis of these results, we propose the following mechanism of activation of resident peritoneal macrophages after low-dose in vivo irradiation: Low-dose irradiation induces the expression of IL-1β mRNA in macrophages, the IL-1β released from the irradiated macrophages stimulating neighboring cells, such as lymphocytes, and by the interaction of the
Fig. 4. Expression of IL-1β mRNA in peritoneal macrophages and IFN-γ mRNA in spleno-lymphocytes obtained from low-dose-irradiated mice. (A) Poly (A)^+ mRNA samples isolated from peritoneal macrophages and spleno-lymphocytes obtained from mice irradiated with 4 cGy were used in the amplification procedure described in “Materials and Methods”. Odd numbered lanes: unirradiated. Even numbered lanes: irradiated with 4 cGy. Lanes 1–4, peritoneal macrophages (primers used: lanes 1 and 2, IL-1β; lanes 3 and 4, β-actin); lanes 5–8, spleno-lymphocytes (primers used: lanes 5 and 6, IFN-γ; lanes 7 and 8, β-actin). (B) Ratios of cytokine to β-actin mRNA expression were determined with a FluorImager. Values, percentages of the sham-irradiation, are expressed as means ± S.D. (n = 4). *p < 0.05 (vs. sham-irradiation).

Fig. 5. Expression of IL-1β mRNA in peritoneal macrophages and IFN-γ mRNA in spleno-lymphocytes exposed in vitro to a low dose of γ-irradiation. (A) Peritoneal macrophages and spleno-lymphocytes were cultured for 24 h then irradiated in vitro with 4 cGy. Poly(A)^+ mRNA was isolated 1 h after irradiation and used in the amplification procedure described in “Materials and Methods”. Odd numbered lanes: unirradiated. Even numbered lanes: irradiated with 4 cGy. Lanes 1–4, peritoneal macrophages (primers used: lanes 1 and 2, IL-1β; lanes 3 and 4, β-actin); lanes 5–8, spleno-lymphocytes (primers used: lanes 5 and 6, IFN-γ; lanes 7 and 8, β-actin). (B) Ratios of cytokine to β-actin mRNA expression were determined with a FluorImager. Values, percentages of the sham-irradiation, are expressed as means ± S.D. (n = 4). *p < 0.05 (vs. sham-irradiation).
activated cells or by their released cytokines, the macrophages are further stimulated. Via this mechanism, slightly activated macrophages would cause production of more activated macrophages which would increase the proliferation of lymphocytes. This proposal is supported by the observed enhancement of accessory function at 4 h, but not 1 h, after in vivo irradiation, as shown in Fig. 1.

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