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Cytokine production by macrophages in association with phagocytosis of etoposide-treated P388 cells in vitro and in vivo

Chizuru Kawagishi, Kahori Kurosaka, Naoko Watanabe, Yoshiro Kobayashi *

Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

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Abstract

Chemotherapy and radiotherapy are performed for cancer patients with the hope that dying cancer cells are safely scavenged by phagocytic cells such as macrophages. In this study, we examined cytokine production by macrophages during and after the phagocytosis of etoposide-treated P388 cells in vitro and in vivo. Etoposide caused apoptosis as early as 5 h after treatment, as assessed as to the exposure of phosphatidylserine, increase in membrane permeability and DNA ladder formation. Phagocytosis by phorbol myristate acetate (PMA)-treated THP-1 cells occurred marginally when P388 cells were treated with etoposide for 10 h, while it occurred significantly with P388 cells treated for 24 h, as evidenced by flow cytometry and confocal microscopy. PMA-treated THP-1 cells produced pro-inflammatory cytokines, such as interleukin (IL)-1α, IL-8 and macrophage migration inhibitory factor (MIF), but not anti-inflammatory cytokines among those tested at the mRNA level during and after the phagocytosis of apoptotic cells. IL-8 and MIF were also produced at the protein level, and the IL-8 production was dependent on cell-to-cell contact when the plasma membranes of apoptotic cells were intact enough not to leak one of the cytoplasmic enzymes, lactate dehydrogenase. In addition, etoposide-treated P388 cells induced neutrophil infiltration as well as MIP-2 production upon injection into the peritoneal cavity of either normal mice or mice with sterile peritonitis. When macrophages ingesting and/or binding apoptotic P388 cells were isolated from the mice with sterile peritonitis using a cell sorter, they were found to produce MIP-2 upon culture.

Keywords: Apoptosis; Macrophage; Etoposide; Phagocytosis; Cytokine

1. Introduction

Chemotherapy and radiotherapy are ideal for killing malignant cells in cancer patients, because such therapies induce apoptosis of these cells [1], and because the therapy-induced apoptotic cells are believed to be safely scavenged by phagocytic cells such as macrophages [2]. Apoptosis is regulated by a set of proteins including p53, whose mutation appears to explain much of the failure of such therapies [3]. Therefore gene therapy to introduce the wild-type p53 has been considered to overcome the lack of induction of apoptosis, although the gene status of p53 is not always highly predictive of the treatment outcome in cancer patients [4].

Since macrophages produce a variety of cytokines in response to bacteria, viruses, tumor cells, and immune complexes, macrophages may also produce cytokines during and after the phagocytosis of chemo-

* Corresponding author. Fax: +81-47-472-7696.
E-mail address: yoshiro@biomol.sci.toho-u.ac.jp (Y. Kobayashi).

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therapeutic agent-treated cells. Identification of the cytokines produced may lead to improvement of the efficacy of these therapies in cancer treatment.

Chemotherapy and radiotherapy commonly cause bone marrow toxicity, which results in a dramatic decrease in the number of circulating neutrophils in particular, and thus granulocyte colony-stimulating factor (G-CSF), for instance, reportedly improves their efficacy by allowing both increases in their doses and decreases in vulnerability to serious infection [5]. Although neutrophils are reportedly critical in cytokine-based gene therapy [6], little attention has been paid to the role of neutrophils as effector cells in combination therapies involving chemotherapeutic agents and G-CSF.

Etoposide is one of the chemotherapeutic agents which are currently used in clinics, and is well known to inhibit topoisomerase II, thereby inducing apoptosis of a variety of cells [7,8]. P388 cells are one of the types of cancer cells which have been employed as a model for assessing the efficacy of chemotherapeutic agents in vitro and in vivo [9]. In this study, we first examined cytokine production by coculturing macrophages with etoposide-treated P388 cells and then examined the in vivo response following intraperitoneal injection of etoposide-treated P388 cells.

2. Materials and methods

2.1. Preparation of cells

A murine leukemic cell line, P388 cells, was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) (Life Technologies, Rockville, MD) and 10^{-5} M 2-mercaptoethanol (culture medium). A murine interleukin (IL)-2-dependent T-cell line, CTLL-2 cells, was maintained in RPMI 1640 medium containing 10% FCS, 5×10^{-5} M 2-mercaptoethanol and 100 U/ml recombinant human IL-2 (kindly provided by Takeda Pharmaceutical Co., Osaka, Japan). A human monocytic leukemia cell line, THP-1 cells, was treated with 160 nM phorbol myristate acetate (PMA) for 72 h at 37°C for differentiation into macrophages, according to the method described previously [10]. P388 cells were adjusted to the cell density of 10^6 cells/ml in the culture medium, followed by the addition of 1 mg/ml of etoposide (Wako, Osaka, Japan) to the final concentration of 1 μg/ml, and then the cells were incubated for 5, 10 or 24 h at 37°C. CTLL-2 cells were washed with prechilled phosphate-buffered saline (PBS) three times, followed by incubation in IL-2-free medium for 28 h at 37°C at the cell density of 5×10^5 cells/ml. To induce necrosis, P388 cells were washed with prechilled PBS three times, followed by adjustment of the cell density to 10^6 cells/ml in PBS. The cells were then subjected to three cycles of freezing at −80°C for 30 min and thawing at 37°C for 5 min. The PMA-treated THP-1 cells were washed with PBS three times, followed by the addition of etoposide-treated P388 cells or necrotic P388 cells, at a ratio of 1:2, and incubation for 3 h at 37°C. As a positive control for cytokine production, PMA-treated THP-1 cells were stimulated with 0.1 μg/ml lipopolysaccharide (*Escherichia coli* O55B5; Difco, Detroit, MI) for 3 h.

2.2. Injection of cells into the peritoneal cavity

CDF1 (6 weeks old, male) mice were purchased from SLC (Shizuoka, Japan). Mice were injected with 2 ml of thioglycollate broth intraperitoneally, and then after 90 h with 2×10^6 of etoposide-treated P388 cells suspended in 2 ml of PBS or 2 ml of PBS intraperitoneally. Normal mice were injected similarly. After 5 h, peritoneal exudate cells (PEC) were collected with prechilled PBS.

2.3. Flow cytometric analysis [10]

The cell size and expression of phosphatidylserine (PS) were analyzed by flow cytometry using a FACScan and Lysys software (Becton Dickinson, Mountain View, CA). The expression of PS was estimated by fluorescein isothiocyanate (FITC)-conjugated annexin V staining. For the phagocytosis assay, etoposide-treated P388 cells were stained with propidium iodide (PI), followed by incubation with PMA-treated THP-1 cells for 3 h at 37°C. The cells were recovered by gentle flushing with prechilled PBS and tapping of the bottom of the plate. Cell infiltration to the peritoneal cavity was examined by flow cytometry. PEC were incubated with Fc block (PharMingen, SanDiego, CA), and then with FITC-conjugated rat anti-mouse Gr-1 (RB6-8C5) antibodies (PharMin-

gen) or FITC-conjugated rat IgG (PharMingen) for 30 min on ice. The cells were then washed twice with PBS and analyzed by flow cytometry.

2.4. Isolation of macrophages ingesting and/or binding apoptotic P388 cells by a cell sorter

Thioglycollate-treated CDF1 mice were injected with 2×10^6 of etoposide-treated P388 cells stained with PI. After 1 h, PEC were collected with pre-chilled PBS, washed with PBS once and stained with FITC-conjugated anti mouse F4/80 antibody (Cosmo Bio, Tokyo) following incubation with Fc block and heat-inactivated mouse serum. F4/80-positive and PI-positive cell population was then sorted with Epics Altra (Beckman Coulter, Tokyo). The cells were observed under confocal microscopy immediately or after a 3-h culture as described below.

2.5. Assaying of DNA fragmentation [11]

Cells were washed with PBS twice, and then lysed with lysis buffer comprising 10 mM Tris–HCl (pH 7.4), 10 mM EDTA and 0.5% Nonidet P-40 for 10 min at 4°C. After centrifugation at 16 000 rpm for 20 min at 4°C, the supernatant was incubated with 0.4 mg/ml of RNase A for 1 h at 37°C, followed by incubation with 0.4 mg/ml of proteinase K for 1 h at 37°C. DNA was precipitated with an equal volume of isopropanol/0.5 M NaCl at −20°C overnight. Samples derived from 1×10^6 cells were analyzed by 2% agarose gel electrophoresis. It should be noted here that only fragmented DNA can be recovered with this method.

2.6. Confocal microscopy [10]

Etoposide-treated P388 cells were stained with PI, followed by culturing with macrophages for 3 h at 37°C. The cells were washed, and adherent cells were recovered as described above. The cells were then stained with FITC-conjugated mouse monoclonal antibody (mAb) to human HLA (ABC) class I (Serotec) for 15 min at 4°C. The cells were examined by confocal microscopy using an InSIGHT plus-IQ system (Meridian Instruments) or Fluoroview system (Olympus).

2.7. Inhibitors

Phospho-L-serine (PLS), RGDS (Arg-Gly-Asp-Ser), and RGES (Arg-Gly-Glu-Ser) were purchased from Sigma (St Louis, MO). PMA-treated THP-1 cells were pretreated with these inhibitors for 30 min at 37°C. Etoposide-treated P388 cells were then added, followed by incubation for 3 h at 37°C, after which cytokine production was evaluated.

2.8. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated from a coculture, and RT–PCR was performed as previously described [10]. For semiquantitation, cDNA was serially diluted, followed by RT–PCR with β_2-microglobulin (β_2-m) and cytokine primers, and polyacrylamide gel electrophoresis. After measurement of the intensity of each band, the results were expressed as percentage of β_2-m. The primers (5′ primer and 3′ primer), annealing temperatures, concentrations of $MgCl_2$, and predicted sizes were previously described, except for MCP-1 and macrophage migration inhibitory factor (MIF). MCP-1: CCTGCTGCTATAACTTCACC, TCAAGTCTTCGGAGTTTGGG, 59°C, 2 mM, 203 bp [12]; MIF: CTGCACAGCATCGGCAAGAT, TTTCTCCCCACCAGAAGGTT, 60°C, 2 mM, 267 bp [13].

2.9. Measurement of human IL-8, human MIF, human IL-1α and mouse MIP-2

After coculturing of macrophages with etoposide-treated P388 cells, the supernatants were harvested. PEC were recovered from mice injected with etoposide-treated P388 cells and centrifuged, followed by harvesting of the supernatants. Samples were stored at −80°C until the assay. The human IL-8 level was determined by means of a specific enzyme-linked immunosorbent assay (ELISA), for which the reagents were provided by Dr. K. Matsushima (University of Tokyo School of Medicine, Tokyo, Japan), as previously described [10]. The detection limit was 10 pg/ml. Human MIF level was determined by means of a specific ELISA comprising biotinylated anti-human MIF antibody and anti-human MIF detection antibody (R&D Systems, Minneapolis, MN). The detec-

tion limit was 10 pg/ml. Human IL-1α level was determined by means of a specific ELISA (Amersham Pharmacia Biotech, UK). The detection limit was 0.1 pg/ml. The mouse MIP-2 level was determined by a specific ELISA according to the method previously described [14]. The detection limit was 20 pg/ml.

2.10. Statistics

The significance of the data was evaluated by means of the Welch method or Student's *t*-test.

3. Results

3.1. Kinetics of the apoptosis of P388 cells induced by etoposide

P388 cells were treated with 1 μg/ml etoposide for various times, and then the exposure of phosphatidylserine (PS), membrane permeability and DNA fragmentation were evaluated. As shown in Fig. 1 (left panels), annexin V-positive cells increased in a time-dependent manner, and a small portion of cells was positive as early as 5 h after treatment. Etoposide also increased the membrane permeability in a time-dependent manner, as shown in Fig. 1 (right panels), as assessed as to stainability with PI. Another indicator of the membrane permeability, trypan blue dye exclusion, showed a gradual loss of membrane intactness, the cell viability being 98 ± 1% at 5 h, 91 ± 1% at 10 h, and 59 ± 5% at 24 h after treatment. Lactate dehydrogenase, one of the cytosolic enzymes, was also detected extracellularly at 10 and 24 h, but not at 5 h (0% at 5 h, 9.6% at 10 h, and 42.8% at 24 h), confirming the gradual loss of membrane intactness. DNA fragmentation was detected 10 h after treatment, although a small level of DNA fragmentation was detected at 5 h (Fig. 2).

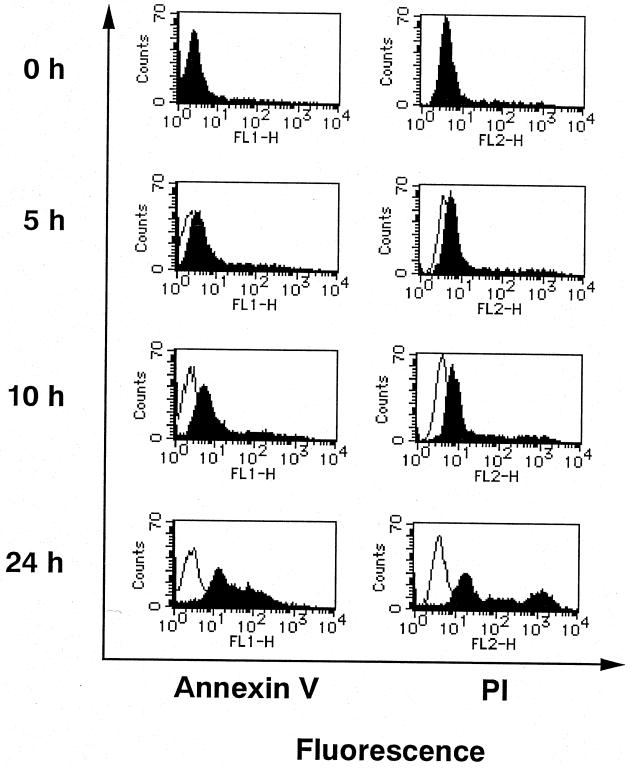

Fig. 1. Kinetics of the apoptosis of P388 cells induced by etoposide. P388 cells were treated with 1 μg/ml etoposide for various times, and then the exposure of PS (left panels) and increase in membrane permeability (right panels) were examined according to the methods described in Section 2. The experimental profiles are shaded, while the control profile is outlined.

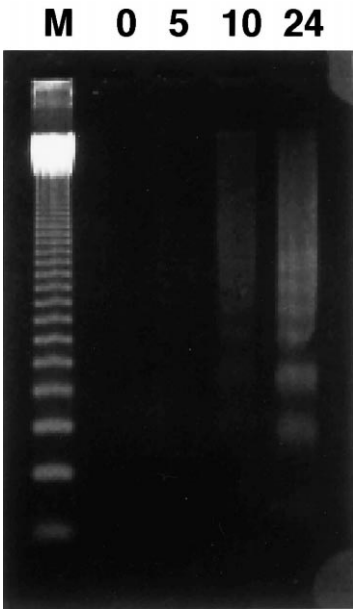

Fig. 2. Kinetics of DNA fragmentation of P388 cells induced by etoposide. P388 cells were treated with 1 μg/ml etoposide for various times, and then DNA fragmentation was examined according to the method described in Section 2. M denotes molecular mass markers.

3.2. Phagocytosis of etoposide-treated P388 cells by PMA-treated THP-1 cells

The human monocytic leukemia-derived cell line,

THP-1, is known to become plastic-adherent and to acquire increased phagocytic ability on treatment with PMA [15]. We therefore examined the phagocytosis of etoposide-treated P388 cells by PMA-treated THP-1 cells by flow cytometry. PI-stained etoposide-treated P388 cells were cocultured with PMA-treated THP-1 cells for 3 h at 37°C. Then the adherent cells were recovered and analyzed by flow cytometry (Fig. 3). The size of adherent cells increased on co-culturing with P388 cells, which had been treated with etoposide for 24 h (Fig. 3G). Furthermore, when cocultured with etoposide-treated P388 cells stained with PI, PMA-treated THP-1 cells became positive, exhibiting red fluorescence, as shown in Fig. 3F,H. Thus, these results indicated that the longer P388 cells were treated with etoposide the more

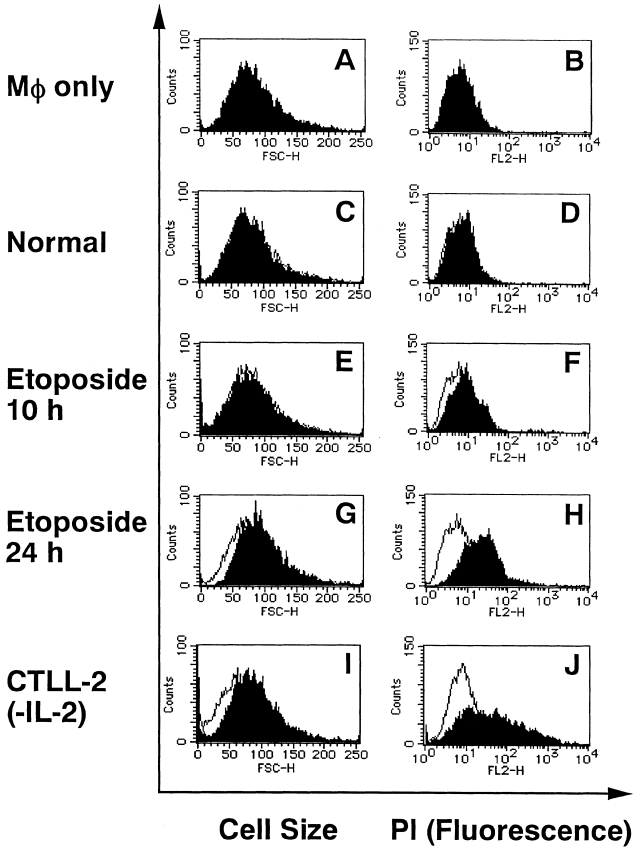

Fig. 3. Phagocytosis of etoposide-treated P388 cells by PMA-treated THP-1 cells. PI-stained P388 cells treated with etoposide for 24 h or PI-stained apoptotic CTLL-2 cells were cocultured with PMA-treated THP-1 cells for 3 h at 37°C as described in Section 2, followed by evaluation of cell size (left panels) and red fluorescence (right panels) by flow cytometry. The experimental profiles are shaded, while the control profile is outlined.

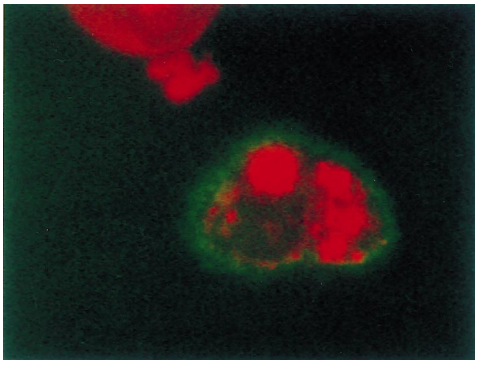

Fig. 4. Confocal microscopic analysis of etoposide-treated P388 cells that have been phagocytosed by macrophages. P388 cells were treated with etoposide for 24 h and then stained with PI, followed by coculturing with macrophages for 3 h at 37°C. The adherent cells were recovered, stained with FITC-conjugated mouse mAb to human HLA (ABC) class I (Serotec) for 15 min at 4°C, and then examined by confocal microscopy.

P388 cells were phagocytosed, and that all the PMA-treated THP-1 cells participated in the phagocytosis.

The phagocytosis of etoposide-treated P388 cells by PMA-treated THP-1 cells was also confirmed by confocal laser microscopy. Here etoposide-treated P388 cells were stained with PI, whereas PMA-treated THP-1 cells were stained with FITC-conjugated mouse mAb to human HLA class I. Fig. 4 clearly demonstrates that PI-stained etoposide-treated P388 cells were engulfed by PMA-treated THP-1 cells.

3.3. *Cytokine production after coculturing of PMA-treated THP-1 cells with etoposide-treated P388 cells*

We then examined the cytokine production after coculturing of PMA-treated THP-1 cells with etoposide-treated P388 cells by means of semiquantitative RT–PCR. The cytokines examined were IL-1α, IL-1β, IL-6, IL-8, tumor necrosis factor-α, MCP-1, MIF, IL-1 receptor antagonist, IL-10, and transforming growth factor-β. The former seven cytokines are pro-inflammatory, whereas the latter three are anti-inflammatory. When PMA-treated THP-1 cells were cocultured with etoposide-treated P388 cells for 3 h, they expressed the mRNAs of three pro-inflammatory cytokines, IL-1α, IL-8 and MIF (Fig. 5A,B). Although other cytokine mRNAs were also examined, none of them were found to be expressed

(data not shown). Coculturing of PMA-treated THP-1 cells with P388 cells treated with etoposide for 24 h produced greater levels of the mRNAs for IL-1α, IL-8 and MIF than that with P388 cells treated with etoposide for 10 h. MCP-1 mRNA was produced only on the coculturing of PMA-treated THP-1 cells with necrotic P388 cells.

The protein levels of IL-8 in the supernatants of cocultures of PMA-treated THP-1 cells with etoposide-treated P388 cells were assayed by means of a specific ELISA, as described in Section 2 (Fig. 6A). The results of IL-8 protein production were essentially the same as those of IL-8 mRNA production in Fig. 5. The protein levels of MIF and IL-1α in the supernatants were also assayed by means of specific ELISAs (Fig. 7 and data not shown). PMA-treated THP-1 cells by themselves produced the similar amount of MIF, 4.4 ± 0.8 ng/ml, as those cocultured with normal P388 cells (Fig. 7), and

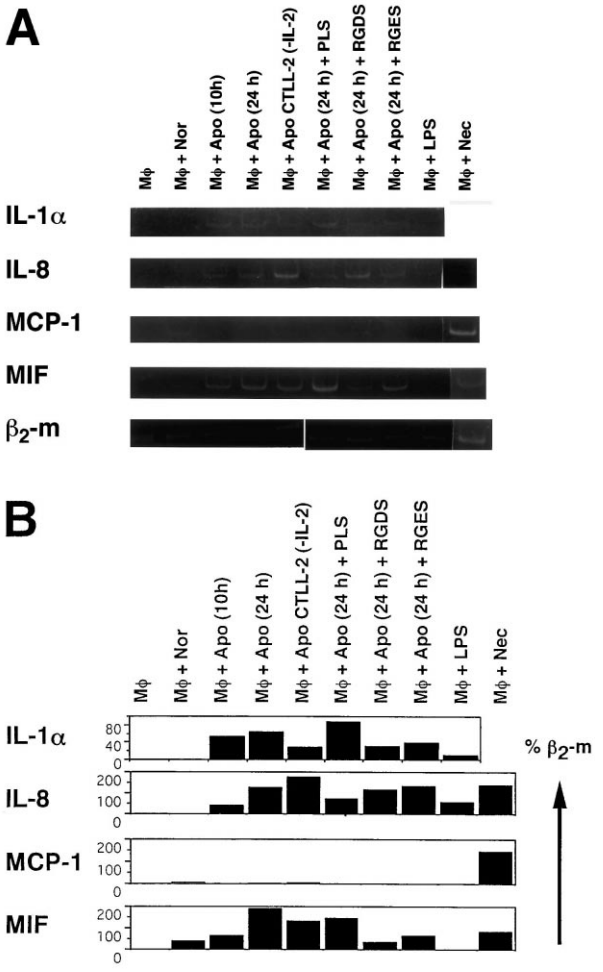

Fig. 5. Cytokine production upon coculturing of PMA-treated THP-1 cells with etoposide-treated or necrotic P388 cells. (A) Polyacrylamide gel electrophoretic pattern of each RT–PCR product. PMA-treated THP-1 cells (macrophages) were cocultured with P388 cells treated with etoposide for 10 or 24 h, necrotic P388 cells, or apoptotic CTLL-2 cells. Total RNA was extracted and subjected to RT–PCR, as described in Section 2. The picture is representative of two independent experiments. (B) The relative amount of each mRNA was determined as described in Section 2, and expressed as a percentage of the amount of β_2-m.

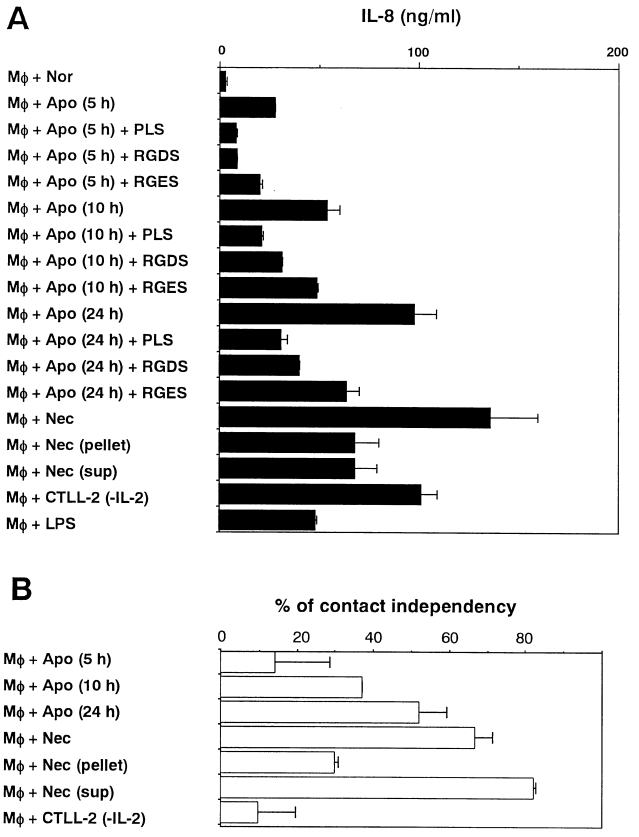

Fig. 6. IL-8 production upon coculturing of PMA-treated THP-1 cells with etoposide-treated or necrotic P388 cells. (A) The levels of IL-8 in the supernatants of cocultures of PMA-treated THP-1 cells (macrophages) with etoposide-treated or necrotic P388 cells were determined by means of a specific ELISA, as described in Section 2. (B) Some cocultures were carried out in a double-chamber culture system (Millipore) to separate macrophages from etoposide-treated or necrotic P388 cells. The experiments were performed in triplicate. IL-8 production with the double-chamber culture system is contact-independent, and the relative contact independency was calculated as the ratios between the IL-8 production with and without the double-chamber culture system. The results are expressed as means ± standard error.

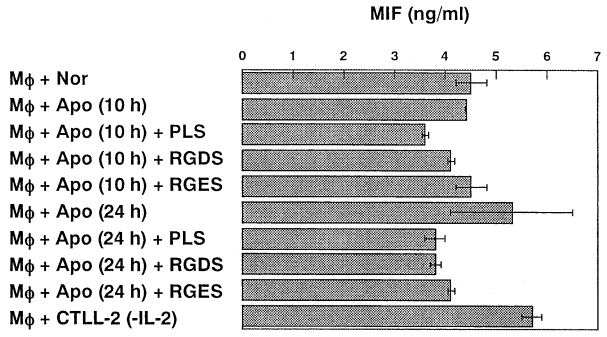

Fig. 7. MIF production upon coculturing of PMA-treated THP-1 cells with etoposide-treated P388 cells. The levels of MIF in the supernatants of cocultures of PMA-treated THP-1 cells (macrophages) with etoposide-treated P388 cells were determined by means of a specific ELISA, as described in Section 2.

MIF protein production was slightly but not significantly increased upon coculturing with apoptotic P388 cells (Fig. 7). IL-1α was not detected in any supernatants. Thus the results of MIF and IL-1α protein production were quite different from those of MIF and IL-1α mRNA production in Fig. 5 (see Section 4).

3.4. Inhibition of cytokine production by either PLS, RGDS or RGES

We then examined whether or not cytokine production was inhibited by either PLS, RGDS or RGES. PLS and RGDS were reported to block the interaction of macrophages with apoptotic cells via the PS receptor and the vitronectin receptor, respectively [16,17]. PLS tended to block IL-8 mRNA production more significantly than RGDS or RGES (Fig. 5B), whereas IL-8 protein production was suppressed by either PLS or RGDS (Fig. 6A). MIF mRNA production was suppressed by either RGDS or RGES more significantly than by PLS (Fig. 5B), whereas MIF protein production was only slightly suppressed by either PLS, RGDS or RGES (Fig. 7). IL-1α mRNA production was enhanced by PLS and suppressed by either RGDS or RGES (Fig. 5B).

3.5. Contact-dependent and -independent production of IL-8

In order to determine whether or not cell-to-cell

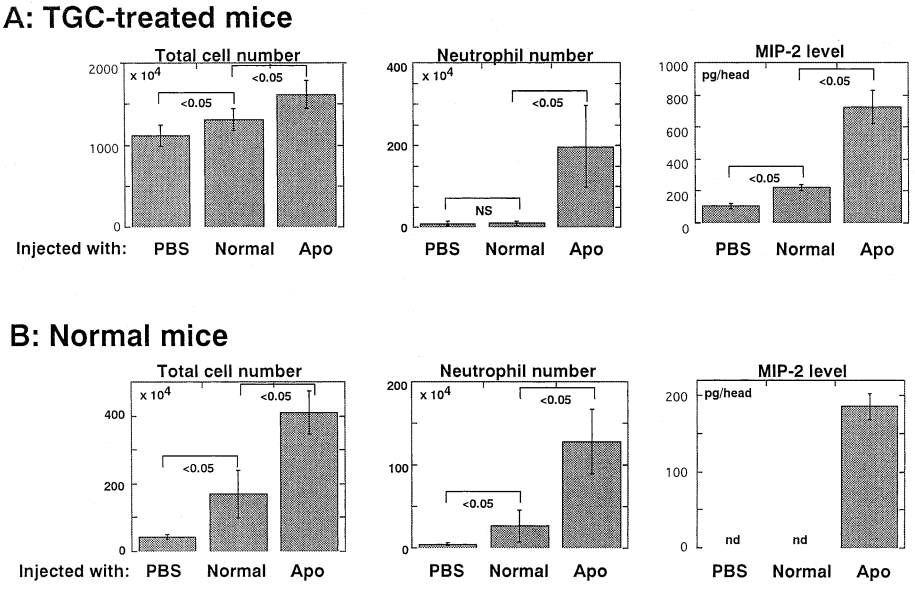

Fig. 8. In vivo response following intraperitoneal injection of etoposide-treated P388 cells. CDF1 mice with sterile peritonitis (A, $n=7$) or normal CDF1 mice (B, $n=6$) were injected with PBS, normal P388 cells, or P388 cells treated with etoposide for 24 h, followed by collection of PEC 5 h later. The total number of PEC, total number of neutrophils, and amount of MIP-2 were determined as described in Section 2. The results are expressed as means ± standard error. nd, not detected. Statistical significance was evaluated either by Welch method (A: MIP-2 for normal cells vs. MIP-2 for apoptotic cells; B: neutrophil number for PBS vs. neutrophil number for normal cells; B: total cell number for normal cells vs. total cell number for apoptotic cells) or by Student's *t*-test (others). NS, not significant.

contact is required for subsequent IL-8 production, we employed a double-chamber culture system for the coculture of etoposide-treated P388 cells with PMA-treated THP-1 cells. IL-8 production in the double-chamber culture system is cell-to-cell contact-independent. As shown in Fig. 6B, IL-8 production by 24 h-treated P388 cells was much more contact-independent than that by 5 h- and 10 h-treated P388 cells, and the contact-independency appears to be reversely related to the intactness of the membranes of apoptotic cells. When necrotic P388 cells were used, IL-8 production was the most contact-independent, as would be expected. We then separated necrotic P388 cells into a pellet and a supernatant, and examined the effect of the double-chamber culture system. The inductive activity in the supernatant as to IL-8 production was much more contact-independent than that in the pellet.

3.6. *In vivo response following intraperitoneal injection of etoposide-treated P388 cells*

We then examined the in vivo response following intraperitoneal injection of etoposide-treated P388 cells into mice with sterile peritonitis or normal mice. Based on the preliminary results, PEC cells were collected 5 h after injection of etoposide-treated or normal P388 cells. As shown in Fig. 8A,B, the numbers of neutrophils were significantly greater than those for normal P388 cells. The levels of MIP-2, a murine homolog of IL-8, in peritoneal exudates paralleled the numbers of neutrophils, although the level of MIP-2 in normal mice was lower than that in thioglycollate-treated mice.

In order to determine whether or not macrophages ingesting apoptotic cells produce MIP-2, we then purified macrophages ingesting and/or binding PI-stained apoptotic P388 cells 1 h after peritoneal injection by a cell sorter, as described in Section 2. Fluorescent microscopy revealed that essentially all macrophages were associated with apoptotic cells, and approximately 17% of macrophages ingested apoptotic cells as evidenced by confocal microscopy (Fig. 9A). There were many macrophages not associated with apoptotic cells in Fig. 9A, probably because confocal microscopy gives a dissected image of a cell at a given point along the *z*-axis. After a 3-h culture, approximately 43% of macrophages ingested apoptotic cells (Fig. 9B). The supernatants taken from such macrophages ingesting and/or binding apoptotic cells were found to contain 40.5 ± 2.8 ng/ml of MIP-2, the level of which was very much close to that obtained by coculturing thioglycollate-induced PEC with apoptotic P388 cells for 3 h, 43.5 ± 0.6 ng/ml.

4. Discussion

This study demonstrated the production of pro-inflammatory cytokines, notably IL-8, by PMA-

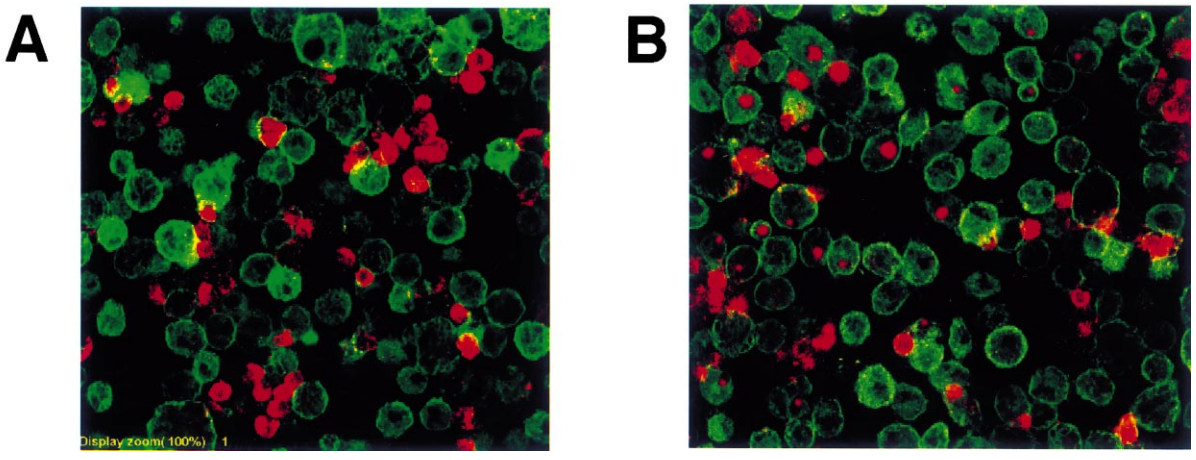

Fig. 9. Confocal microscopic analysis of macrophages ingesting and/or binding apoptotic P388 cells. P388 cells were treated with etoposide for 24 h and then stained with PI, followed by peritoneal injection. One hour later, the peritoneal exudate cells were recovered, stained with FITC-conjugated anti F4/80 antibody, and then PI-positive and F4/80-positive cells were sorted, followed by observation under confocal microscopy immediately (A) or after a 3-h culture (B).

treated THP-1 cells during and after the phagocytosis of etoposide-treated P388 cells. Moreover, intraperitoneal injection of etoposide-treated P388 cells into mice with sterile peritonitis and normal mice induced neutrophil infiltration and MIP-2 production. When macrophages ingesting and/or binding apoptotic P388 cells were purified by a cell sorter, they were found to produce the similar amount of MIP-2 upon further culture as those cocultured with apoptotic P388 cells in vitro, further supporting that these two findings in vitro and in vivo are intimately correlated.

During treatment of P388 cells with etoposide, membrane intactness was gradually lost, and the IL-8 production became partly independent of cell-to-cell contact. Moreover, necrotic P388 cells also induced the production of IL-8, which was mediated mainly by a cell-to-cell contact-independent mechanism. Therefore, the production of large amounts of pro-inflammatory cytokines observed in this study is likely to be induced by either late apoptotic or secondary necrotic cells, which occur under pathological conditions such as ischemia reperfusion injury, although P388 cells treated with etoposide for 5 h, i.e., early apoptotic cells, induced the production of a smaller but significant level of IL-8. This observation may be related to neutrophil infiltration following apoptosis [18,19].

This study focused on cytokine production by macrophages during and after the phagocytosis of etoposide-treated P388 cells in vitro and in vivo. Etoposide and X-irradiation are known to cause bone marrow toxicity, thereby reducing the number of neutrophils. Since we recently found that X-irradiation induced apoptosis in the thymus as well as transient infiltration of neutrophils [19], it is possible that etoposide induces similar responses in mice bearing P388 cells. In support of this possibility, neutrophil infiltration was induced not only in mice with sterile peritonitis but also in normal mice by intraperitoneal injection of etoposide-treated P388 cells. If a similar response does occur in tumor bearing mice in response to chemotherapeutic agents, what role might infiltrating neutrophils play? In bronchioloalveolar carcinomas, tumor-derived IL-8 is reportedly associated with a high risk of death [20]. In contrast, neutrophils are reportedly critical in cytokine-based gene therapy [6]. Thus future study should reveal whether or not the activation of infiltrating neutrophils may be beneficial for cancer treatment.

Among the seven pro-inflammatory cytokines examined, IL-1α, IL-8 and MIF mRNAs were detected in cocultures of etoposide-treated P388 cells with macrophages. Although we also examined production of IL-1α and MIF proteins, IL-1α was not detected in any culture supernatants and MIF production was only slightly enhanced by coculturing with apoptotic cells. The failure of protein production may be explained by that these two cytokines are known to have no signal peptide for secretion. So the biological significance of the production of IL-1α and MIF mRNAs is not known at present. However, MIF is reported to be capable of functionally inactivating p53 [21], and therefore the production of MIF mRNA by macrophages in response to either apoptotic cells or necrotic cells, which may occur in a chronic inflammatory disease, may strengthen the link between inflammation and tumorigenesis, as suggested [21].

Interestingly, MCP-1 mRNA was detected in a coculture of necrotic P388 cells, but not etoposide-treated P388 cells, with macrophages, supporting the notion that necrotic and apoptotic cells are not the same in terms of the biological response induced by them. If the number and/or capacity of macrophages are not sufficient to remove apoptotic cells, apoptosis may advance further to give rise to secondary necrotic cells. Consequently it is reasonable to assume that necrotic cells induce the infiltration of macrophages through MCP-1, because MCP-1 is a chemotactic factor for monocyte and macrophages [22]. Presumably because P388 cells treated with etoposide for 24 h were not completely necrotic, these cells might not have induced MCP-1 production.

In conclusion, this study demonstrated a novel response during and after the phagocytosis of etoposide-induced apoptotic P388 cells in vitro and in vivo, and suggested that neutrophils may infiltrate into the sites where chemotherapeutic agents induce massive apoptosis.

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