Interferon-γ-Dependent Expression of Inducible Nitric Oxide Synthase, Interleukin-12, and Interferon-γ-Inducing Factor in Macrophages Elicited by Allografted Tumor Cells

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Macrophages (Møs) play a crucial role in the defense against tumors and parasites (1). Mø activation has been defined as a response of Møs to the stimulation by Mø-activating factors including IFN-γ; and the response includes the production of cytokines and cytotoxicity towards tumor cells and parasites. One of the main cytokines produced by activated Møs is IL-12, which is the determining factor in the development of the T cell response (2). L-Arginine-derived nitric oxide (NO), a soluble cytotoxic molecule, is also produced in activated Møs by inducible NO synthase (iNOS) (3, 4). Several reports have shown that in vitro-activated Møs kill some tumor cells (5-8) and bacteria (9,10) by an NO-dependent mechanism that can also work in the absence of cell-to-cell contact (5, 7). The antimicrobial activity of NO has been reaffirmed with the generation of iNOS mutant mice, which animals show altered responses to bacterial infection (11, 12). In addition, a new cytokine, IFN-γ-inducing factor (IGIF), which induces IFN-γ production in T cells, was recently reported to be expressed in activated Møs (13). IL-12 primes Møs for NO production in vivo (14), whereas the IL-12 p40 gene promoter is primed by IFN-γ in monocytic cells (15). These findings imply that the signals for Mø activation might be initiated by IGIF and followed by IFN-γ, IL-12, and iNOS expression.

Møs are the major population of cells infiltrating into the rejection site in skin or organ allografts, and may play a role in the process of allograft destruction (16). The obstacle to study of the mechanisms of allograft rejection seems to reside in the technical difficulty in...
harvesting host cells infiltrating into the grafts. Therefore, we have developed a model system in which 3-methylcholanthrene-induced ascites type fibrosarcoma (Meth A) was transplanted into the peritoneal cavity of allogeneic C57BL/6 mouse. Since Meth A cells are an ascites type, the advantage of this system is that we can obtain almost all of the cells infiltrating into the rejection site simply by lavage of the peritoneal cavity (17-21). In this system, we found that Meth A-Møs were the major cytotoxic cells against Meth A tumor cells in vitro (19) and in vivo (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the in vivo (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the in vivo (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the in vivo (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the in vivo (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the in vivo.

MATERIALS AND METHODS

Preparation of Møs. Møs were isolated from male, 7-12-week-old, specific pathogen-free C57BL/6 mice (Japan SLC, Hamamatsu, Japan) or 9-week-old C57BL/6 IFN-γ−/− mice (Jackson Lab., Bar Harbor, ME). For Meth A-Mø isolation each mouse was injected i.p. with 3 × 10⁶ Meth A cells, which were kindly provided by Dr. S. Muramatsu, Dept. of Zoology, Faculty of Science, Kyoto University, Japan. At various time intervals after the transplantation Meth A-Møs were isolated as described (17-21) with some modifications. After the leukocyte-rich fraction had been obtained, Møs were separated from it by a discontinuous density gradient of Percoll (Pharmacia, Uppsala, Sweden). Resident-Møs and BCG-Møs were collected by i.p. lavage of untreated mice and mice that had received an i.p. injection of 10⁷ CFU of Mycobacterium bovis BCG per mouse 5 days earlier, respectively. The Møs thus obtained were washed twice with RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum (Cell culture Labs., Cleveland, OH) and antibiotics, and plated. The Møs were further purified by removing the non-adherent cells by washings with warm phosphate-buffered saline after 2 h of incubation at 37 °C in 5% CO₂. Adherent cells were Møs with >90% purity as judged by Mac-1 staining and by morphological criteria. For Western blot analysis and RNA isolation, the cells were seeded at 2 × 10⁶ cells/ml in 9 cm-diameter dishes (Falcon, Lincoln Park, NJ); and for NO₂ accumulation, ⁵¹Cr release, and DNA fragmentation assays, the cells were seeded at 2 × 10⁵ cells/well in the lower chamber of a Transwell 24-well plate (Costar, Cambridge, MA).

SDS-PAGE and Western blot. SDS-PAGE was carried out following Laemmli’s method (22). After finishing the SDS-PAGE, proteins were transferred onto 0.2 µm pore-size nitrocellulose membranes (Schleicher & Schuell, Tokyo, Japan) in 25 mM Tris buffer (pH 8.3) containing 192 mM glycine and 20% methanol. The membrane was blocked for 30 min at room temperature with 1% BSA in TBST buffer [25 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.05% NaN₃, and 0.2% Tween 20] at room temperature for 30 min. The membrane was then incubated with rabbit anti-mouse iNOS antibody (Transduction Laboratories, Lexington, KY) at a 1:1000 dilution for 1 h at room temperature with a 1:5000 dilution of donkey anti-rabbit IgG antibody conjugated to alkaline phosphatase (Chemicon, Temecula, CA), washed again, and developed.

NO₂ accumulation. NO₂ accumulation was assayed by the Griess reaction (23). An equal volume of Griess reagent was added to 50 µl of each culture supernatant, and the amounts of NO₂ were estimated by reading the OD₄₅₀nm against a standard curve of NaNO₂.

⁵¹Cr release assay. After removal of the non-adherent cells from the lower chamber of the Transwell, 800 µl of RPMI 1640 medium was added to the chamber, and prewetted Transwell inserts were placed in the wells. An inhibitor of iNOS, N⁶-monomethyl-L-arginine (N-MMA; Calbiochem, La Jolla, CA), was also included when indicated. P815 cells were collected during the logarithmic phase of growth and 10⁷ cells/ml were labeled in medium with 1 µCi/ml of Na₃[¹⁵Cr]O₃ (DuPont New England Nuclear, Boston, MA) for 2 h at 37 °C. The cells were then extensively washed and 6.6 × 10⁴ cells in 200 µl of medium were added onto the insert. After an 18-h incubation at 37 °C in 5% CO₂, aliquots of the supernatants were collected and counted for ⁵¹Cr or frozen at −70 °C for later measurements of NO₂ accumulation as described above. The percent specific ⁵¹Cr release was calculated as follows: % specific ⁵¹Cr release = [(Experimental cpm- Spontaneous cpm)/(Total cpm-Spontaneous cpm)] × 100. Total release of ⁵¹Cr was determined by lysis with 0.5% Triton X-100. Spontaneous release was determined in the absence of Møs and was ≤ 25%.

DNA fragmentation. DNA fragmentation was assayed as described (5) with some modifications. P815 cells in the logarithmic phase of growth were labeled in medium with 0.04 µCi/ml [methyl ³H] thymidine (DuPont New England Nuclear).
FIG. 1. Expression of iNOS, IL-12, and IGIF mRNAs in Meth A-Ms. The expression of iNOS, IL-12, IGIF, and G3PDH (as a control) mRNAs was estimated by RT-PCR. Lanes 1-9 correspond to 0 h (resident-Ms), 6 h, 1, 3, 5, 7, 11, 13 and 18 days, respectively, after Meth A transplantation.
FIG. 2. Time-dependent expression of iNOS and nitrite production in Meth A-Møs. (A) SDS-PAGE of cell lysates (each 10 µg protein) of Meth A-Møs followed by Western blotting with anti-iNOS IgG antibody. The numbers on the lanes represent the days after Meth A cell transplantation. Relative molecular masses of prestained markers are shown on the left. Arrow indicates the specific iNOS doublet (~130 kDa). Results are a representative of three experiments. (B) Nitrite production in Meth A-Møs. The values are the mean ± SD of triplicate experiments.

iNOS protein expression in Meth A-Møs was detected by immunoblot on days 5-9, reaching its peak on day 7. A similar pattern was obtained for the concentrations of NO\textsubscript{2} released in vitro by Meth A-Møs isolated during the course of the rejection of Meth A cells (Fig. 2B).

Impairment of iNOS, IL-12, and IGIF induction in Meth A-Møs or BCG-Møs from IFN-γ−/− mice. We previously reported that the leukocytes infiltrating into the rejection site after Meth A transplantation released IFN-γ (18), one of the main cytokines that induce iNOS (3, 4) and IL-12 (2, 14) in Møs. Fig. 3 shows that the iNOS and IL-12 mRNA expression was impaired in Meth A-Møs from C57BL/6 IFN-γ−/− mice. Unexpectedly, the expression of IGIF mRNA was also completely suppressed in Meth A-Møs from IFN-γ−/− mice (Fig. 3). Similarly, these mRNAs were hardly expressed in the BCG-Mø, another well-known in vivo-activated Mø, from C57BL/6 IFN-γ−/− mice (Fig. 3), indicating that IFN-γ was essential for the iNOS, IL-12, and IGIF expression in the in vivo-activated Møs.

NO-mediated DNA fragmentation of P815 cells and 51Cr release from the targets caused by Meth A-Møs. The activation of iNOS produces an L-arginine-derived NO, which has been demonstrated to be a soluble effector molecule cytotoxic against some tumor cells (5-8). Next, we tested the role of NO released in vitro by Meth A-Møs to assess whether or not the in
**FIG. 3.** Impairment of iNOS, IL-12 and IGIF induction in Meth A- or BCG-Mφs from IFN-γ−/− mice. The expression of iNOS, IL-12, IGIF and G3PDH (as a control) mRNAs was estimated by RT-PCR in Meth A-Mφs (on day 7 after Meth A cell transplantation) or BCG-Mφs (on day 5 after BCG injection) in C57BL/6 IFN-γ+/+ and IFN-γ−/− mice. Results are representative of two experiments.

**vivo** activation of Meth A-Mφs correlated with its cytotoxicity **in vitro.** We used P815 cells, a donor-type and NO-sensitive tumor cell line (5), as target cells. Meth A-Mφs and P815 cells were cultured in different compartments of a Transwell (see ‘‘Materials and Methods’’), separated by a cell-impermeable membrane (insert) to prevent cell-to-cell contact. Fig. 4A shows the release of 51Cr from P815 cells after an 18 h-incubation with Meth A-Mφs. Under the conditions used, the fragmentation of [3H]thymidine-labeled DNA of P815 cells was also detected (Fig. 4B). The extent of DNA fragmentation (27.4%) was higher than that of 51Cr release from P815 cells (17.3%), implying the DNA fragmentation preceded plasma membrane damage. Both the cytotoxicity of Meth A-Mφs against P815 cells and the DNA fragmentation of the targets were inhibited by the addition of N-MMA, an iNOS inhibitor, to the culture medium (Fig. 4A and B), suggesting that these actions were mediated by NO released by Meth A-Mφs. In fact, when the NO2 concentration in other samples from the same wells was measured, the release of NO2 from Meth A-Mφs was also blocked by N-MMA (Fig. 4C). As expected, Meth A-Mφs isolated from IFN-γ−/− mice neither lysed P815 cells nor released NO (data not shown).

**NO-mediated ‘‘DNA ladder’’ formation in P815 cells.** To determine whether the DNA fragmentation of P815 cells detected by the use of [3H]thymidine conformed to the pattern of oligonucleotides with molecular weights of 180-200 base pairs, a characteristic of apoptotic death, we cultured Meth A-Mφs and P815 cells in different compartments of Transwell microtiter plates. The DNA extracted from P815 cells showed the characteristic ‘‘ladder’’ appearance that is a hallmark of apoptosis (Fig. 5, lane 3). The DNA fragmentation of P815 cells was also inhibited by the addition of N-MMA to the medium (Fig. 5, lane 4).

**DISCUSSION**

This study has shown that iNOS, IL-12, and IGIF mRNAs were expressed transiently in Meth A-Mφs during the rejection of allografted Meth A cells in C57BL/6 mice. The iNOS protein expressed in Meth A-Mφs showed a characteristic dimer (of ≈130 kDa) similar to that of iNOS purified from the RAW 264.7 Mφ cell line (24). The peaks of iNOS and IL-12 mRNA expression in Meth A-Mφs coincided in time with the maximal cytotoxic activity of Meth A-
FIG. 4. Inhibitory effects of N-MMA on cytotoxicity of Meth A-Møs against P815 tumor cells (A), DNA fragmentation of the target cells (B), and nitrite production by Meth A-Møs (C). Meth A-Møs isolated as described in Fig. 2 were added to the lower chamber of a Transwell. P815 cells labelled with $^{51}$Cr (A) or $[^3H]$thymidine (B) were added within the insert, and they were cultured in the presence or absence of 0.5 mM NMMA for 18 h. Panel C shows the nitrite concentration measured using aliquots from the same samples. Results are representative of five experiments.

Møs against Meth A cells (19) and with the maximal release of IFN-γ from the infiltrating cells (18). Therefore, Meth A-Møs induced in C57BL/6 mice 7 days after i.p. transplantation of allogeneic Meth A tumor cells are thought to be a type of highly activated Møs. The expression of iNOS, IL-12, and IGIF mRNAs in Meth A- or BCG-Møs was impaired in C57BL/6 IFN-γ −/− mice (Fig. 3), demonstrating the essential role of IFN-γ in the expression of iNOS, IL-12, and IGIF mRNAs in these in vivo-activated Møs. Similarly, the essential role of IFN-γ in the iNOS expression was earlier demonstrated by others using mice whose encoding genes for IFN-γ (25), one chain of IFN-γ receptor (26), or IFN regulatory factor (27) had been disrupted or by the injection of monoclonal antibody against IFN-γ (8). Also, since the IL-12 p40 gene promoter is known to be primed by IFN-γ in monocytic cells (15), it is reasonable that the expression of IL-12 in Meth A-Møs would be IFN-γ dependent (Fig. 3). Unexpectedly, however, the expression of IGIF mRNA in Meth A-Møs was also IFN-γ dependent (Fig. 3), although IGIF had been reported to induce IFN-γ production in T cells (13).
FIG. 5. NO-mediated DNA ladder of P815 cells. Meth A-Møs prepared as described in the legend to Fig. 2 were added to the lower chamber of a Transwell, and P815 cells were added onto the insert. After an 18 h-incubation, the DNA from P815 cells was extracted and subjected to electrophoresis. Lane 1, standard 123-base pair DNA ladder; Lane 2, DNA from P815 cells cultured in the absence of Meth A-Møs; Lane 3, DNA from P815 cells cultured in the presence of Meth A-Møs; and Lane 4, DNA from P815 cells cultured in the presence of Meth A-Møs and 0.5 mM N-MMA.

Meth A-Møs killed donor-type, NO-sensitive P815 cells in vitro by a mechanism that fulfill the criteria for apoptotic cell death. The extent of Meth A-Møs-induced DNA fragmentation of P815 cells was higher than that of $^{51}$Cr release from the targets, implying that these two processes are sequential in time. The apoptotic type of cell death is a process that starts with fragmentation of genomic DNA into small oligomers, followed by the release of intracellular contents in the later stages (28). Both the $^{51}$Cr release and the DNA fragmentation did not require cell-to-cell contact, and both were inhibited by N-MMA, an iNOS inhibitor. In addition, N-MMA inhibited the accumulation of NO$_2^-$ in Meth A-Møs. These results demonstrate that Meth A-Møs killed P815 cells through NO as the soluble effector molecule in agreement with previous experiments that used in vitro-activated Møs (5, 7).

The findings that the time-course of the expression of iNOS mRNA in Meth A-Møs correlated with that of the rejection of Meth A cells by the mice (19), and that Meth A-Møs lysed P815 cells in an in vitro assay by an NO-dependent mechanism, could lead us to speculate that NO is involved in the rejection of Meth A cells. However, in vitro experiments showed that Meth A cells were insensitive to NO released from Meth A-Møs (data not shown). The role of NO in allograft rejection is somewhat controversial: Bastian et al. (29) have shown that the inhibition of NO production did not affect the rejection of cardiac allografts in mice. In contrast, Worral et al. (30), using the same cardiac allograft system, showed that treatment with aminoguanidine, an another inhibitor of iNOS, prolonged the graft survival. Since NO also acts as a scavenger of the superoxide anion released from polymorphonuclear leukocytes (31) and suppresses T cell-mediated reactions and lymphocyte proliferation (32-35), these biological functions rather than cytotoxic activity of NO may have enhanced allograft survival. Cytotoxic effects of NO released from Møs on Møs themselves have also been reported (36).

In conclusion, our results show that Meth A-Møs expressed iNOS, IL-12, and IGIF mRNAs during the rejection of allografted Meth A tumor cells in mice. At the peak of their expression,
Meth A-Møs appear to be a type of in vivo-activated Møs that can kill P815 cells through apoptotic cell death by a cell-to-cell contact-independent and NO-dependent mechanism. IFN-γ was essential not only for the expression of iNOS and IL-12 but also for the expression of IGIF in the in vivo-activated Møs (e.g., Meth A-Møs and BCG-Møs), implying that IFN-γ, but not IGIF, would be the signal molecule to initiate the activation of Møs in vivo.

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REFERENCES