Apoptotic Cell Effects on Nitric Oxide Production in Activated Macrophages

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Abstract

Macrophages can be activated to kill tumor necrosis factor (TNF)-sensitive, nitric oxide (NO)-resistant 3T3-VSV and NO-sensitive, TNF-resistant P815 cells. Activated macrophages co-cultivated with 3T3-VSV but not P815 targets show decreased production of NO. Since TNF can induce apoptosis and apoptotic cells can interfere with macrophage activation, apoptotic cells were compared to normal cells for their ability to decrease NO production. Activated RAW 264.7 cells were incubated with medium alone, or medium containing apoptotic or normal CTLL-2 cells and, the NO concentration of the supernatants assayed. Apoptotic but not normal CTLL-2 cells decreased NO production by activated macrophages.

Introduction

Macrophages can be activated by exposure to a mixture of interferon and lipopolysaccharide (LPS) to kill both tumor and virally-infected cells (1). Macrophages can use various cytotoxic mechanisms to kill virally infected and tumor target cells, including tumor necrosis factor-alpha (TNF) (2) and nitric oxide (NO) (3). Some target cells, such as P815 cells, are sensitive to NO but not to TNF while others, such as vesicular stomatitis virus-infected BALB/c3T3 cells (3T3-VSV), are killed by TNF but not NO (3). TNF is produced early after macrophage activation while NO, which can be toxic to the macrophages themselves, is produced much later (4). Incubation of activated macrophages with VSV-3T3 targets causes a decrease in NO-production compared to activated macrophages incubated with either P815 targets or medium alone (Fig. 1, and [3]). PCR analysis of inducible NO synthase (iNOS) gene showed this decrease is due to a decreased level of mRNA for the enzyme iNOS after both 8 h and 16 h (Fig. 2, and [3]). Inflammation is the body's normal response to injury and is triggered by the appearance of intracellular contents in the extracellular environment. Apoptosis is a mechanism of programmed cell death by which the body can rid itself of unwanted cells without inducing an inflammatory response (5). Since some viral infections (6-8) and exposure to TNF (9-11) can cause cells to become apoptotic and exposure of macrophages to apoptotic cells has been reported to affect macrophage function (12), we hypothesize that VSV-3T3 cells become apoptotic after interaction with macrophage produced TNF, and that exposure to apoptotic cells cause a decrease in NO-production. To test this hypothesis, we generated apoptotic CTLL-2 cells by culturing these IL-2 dependent cells in the absence of IL-2 and assaying NO production by activated macrophages with normal and apoptotic CTLL-2 target cells. We monitored the induction of apoptosis by assaying the caspase activity of the target cells.

Materials and Methods

Tissue Culture

Macrophage cell line RAW 264.7 ATCC TIB71 (13) was cultured in MEM consisting of Eagle's modified minimum essentials medium (Hyclone, Logan, UT) supplemented to 15mM HEPES and 10% fetal bovine serum (FBS) (Atlanta Biologicals). CTLL-2, a lymphocyte cell line ATCC TIB 214 (14), was cultured in RPMI 1640 medium (Hyclone, Logan, UT) supplemented to 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 5 μM 2-mercaptoethanol (all from Sigma, St. Louis, MO), 10% FBS, and 5% Rat-Stim with Concavalin A (ConA) (BD Biosciences). All incubations were in a moist atmosphere of 5% carbon dioxide in air at 37°C.

CTLL-2 Caspase 3 Activity Assay

CTLL-2 cells were cultured at 7 x 10^4 cells/ml in 20 ml in RPMI 1640 with Rat-Stim as a source of IL-2 overnight and then washed and cultured in RPMI 1640 plus ConA (Sigma, St. Louis, MO) for 0, 4, 6, 8, or 10 h at 37°C. After incubation, cells were prepared for caspase 3 assay.

Caspase 3 Assay

Caspase 3 assay was performed with caspase 3 assay kit from Sigma following the supplier's directions (15). CTLL-2 cells cultured as previously described, washed in PBS and 2 x 10^6 cells were lysed with lysis buffer provided in the kit for 10 min. The lysates were centrifuged at 16,000 – 20,000 g for 10 min. The supernatants were stored at –80°C until assay. Cell lysates were placed in a 96-well plate with the caspase 3 substrate acetyl-Asp-Glu-Val-Asp *p*-nitroaniline. The caspase activity was measured by determining the optical density of each well at 405 nm using spectrophotometer and *p*-nitroaniline as a standard. The activity is expressed as μM per 2(10)^6 cells.

Nitric Oxide Assay

Macrophages were activated with LPS (10 ng/mL) and recombinant rat IFN-gamma (Amgen, Thousand Oaks, CA, 5 units/mL) in MEM for 4 h. Activators were washed off and monolayers were washed twice before addition of target cells. CTLL-2 cells were cultured at 1 x 10^4 cells/mL in RPMI 1640 with standard supplements with Rat-Stim or without Rat-Stim but with CON A (1 μg/ml) for 6 h. Cells were washed once in mPBS and resuspended to 2 x 10^5 in MEM. CTLL-2 cells cultured with or without Rat-Stim for 6 h were added to activated RAW 264.7 cells and incubated in a moist atmosphere of 5% carbon dioxide in air at 37°C for 16 hrs.

The amount of nitric oxide produced was determined by measuring the amount of nitrite in culture supernatants. Cell supernatants were mixed with equal volume Griess reagent and the resultant color measured at 540 nm using a spectrophotometer (16). The amount of nitric oxide was calculated using a standard curve of sodium nitrite and expressed as μM per 2.5(10)^5 macrophages.

Results and Discussion

In order to determine whether apoptotic cells decrease NO production by activated macrophages, we first determined when CTLL-2 cells cultured without IL-2 became apoptotic by performing a caspase 3 assay. Caspase 3 was selected as an indicator for apoptosis because it is a common effector caspase that is activated no matter how apoptosis is induced (17). CTLL-2 cells cultured without IL-2 for 6 and 8 h showed an increase in caspase 3 activity, while cells cultured without IL-2 for 10 h showed a decrease in caspase 3 activity (Fig 3). To test if apoptotic cells cause a decrease in NO production by activated macrophages, CTLL-2 cells were cultured without IL-2 for 6 h and added to activated macrophages. Culture supernatants of macrophages co-cultured with CTLL-2 cultured without IL-2 had a lower concentration of NO than supernatants of macrophages cocultured with CTLL-2 cells cultured with IL-2 or with medium alone (Table 1).

Since the decreased NO production by macrophages with 3T3-VSV cells is due to a decreased level of mRNA for the enzyme inducible nitric oxide synthase (13), future work will test if the presence of apoptotic cells also causes a decreased expression of the inducible nitric oxide synthase gene.

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Acknowledgements

This work was supported by a Howard Hughes Medical Institute Undergraduate Research Grant to The University of Alabama and the laboratory of Dr. Paul LeBlanc in the UA Department of Biological Sciences.

Figure and Table Legends

Figure 1. The effect of target cells on the level of nitrite and IL-6 in the supernatant of activated macrophages. Macrophages produced less NO with 3T3-VSV targets than P815 targets. There were no significant differences in IL-6 activity produced with each target.

Figure 2. PCR analysis of iNOS and IL-6. Reverse transcriptase PCR shows less iNOS mRNA in activated macrophages with 3T3-VSV targets than with P815 targets at both 8 h (A) and 16 h (B). There was no significant difference in IL-6 mRNA in activated macrophages.

Figure 3. CTLL-2 cells were cultured without IL-2 and caspase 3 activity was assayed and expressed as μM pNA released per 2(10)^6 cells.

Table 1. Activated macrophages were cultured with targets and NO production was assayed and expressed as μM per 2.5(10)^5 macrophages. Cell viabilities were determined by trypan blue exclusion count.

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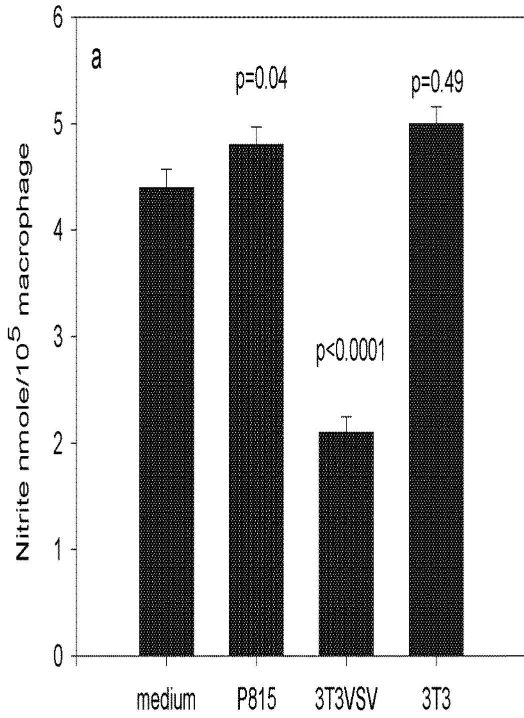


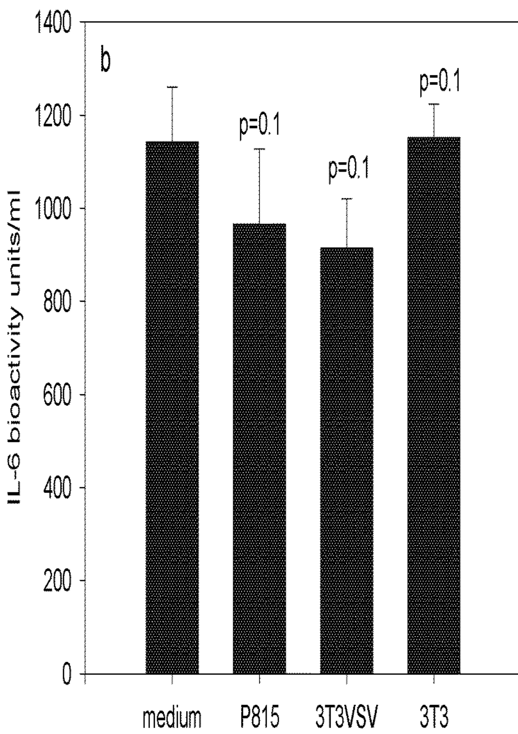
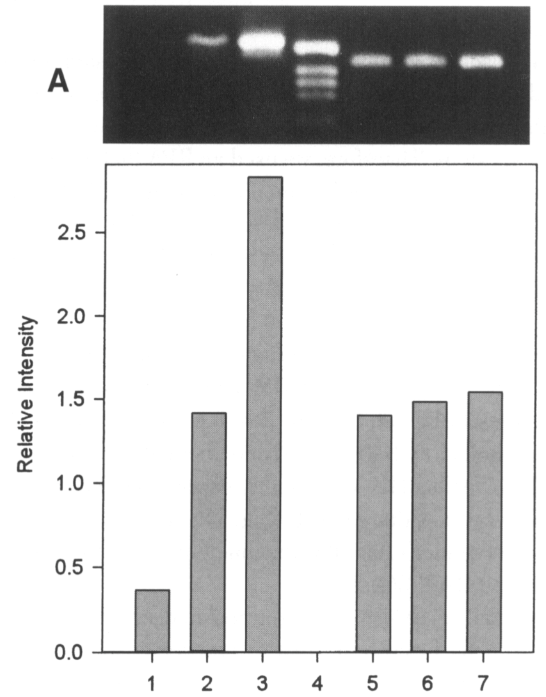


Figure 1: NO and IL-6 Production of Activated Macrophages with 3T3-VSV and P815 Targets



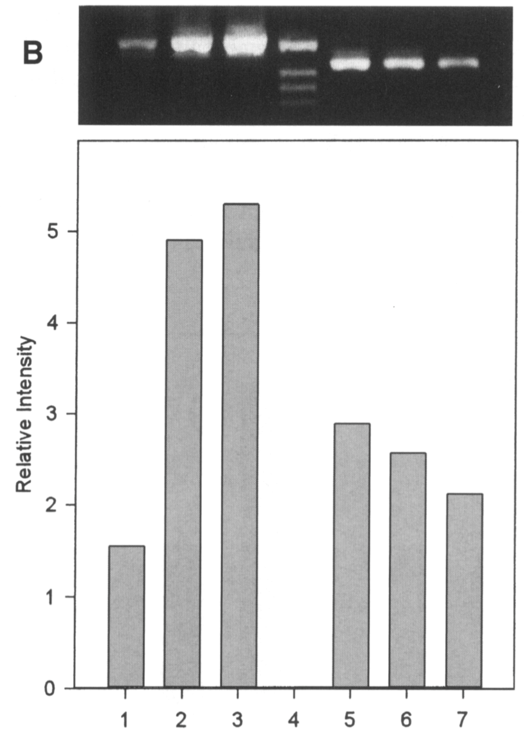


Figure 2: 3T3-VSV cells reduce macrophage iNOS mRNA level but not IL-6

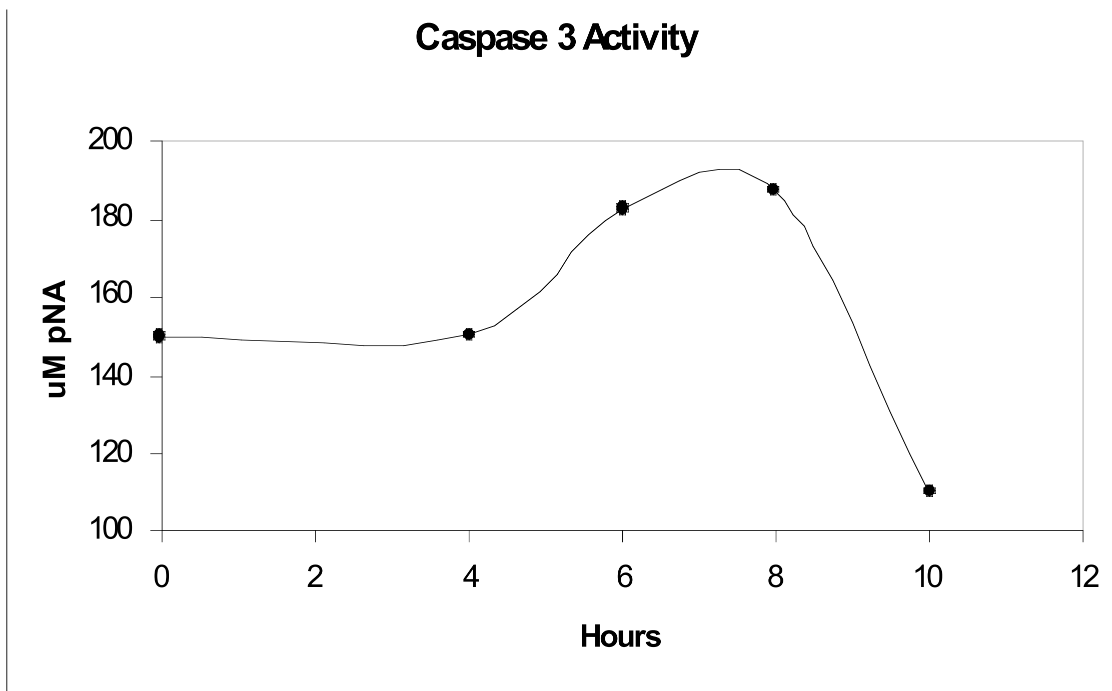


Figure 3: Caspase 3 Activity of Apoptotic Cells

Activated macrophages cocultured with	NO in culture supernatants	Target Cell Viability (percent)
Medium	4.91(± .32)	NA
CTLL-2 Cultured with IL-2	4.73(± .22) P=.13	98
CTLL-2 Cultured without IL-2	4.39(± .28) P<.01	95

Table 1: NO Production by Activated Macrophages