Pergamon

0960-0760(95)00182-4

*J. Steroid Biochem. Molec. Biol.* Vol. 55, No. 3/4, pp. 291–296, 1995

0960-0760/95 $9.50 + 0.00

# Cytotoxic Actions of Cytokines on Cultured Mouse Luteal Cells are Independent of Nitric Oxide

**Toushun Jo,[1,2]* Nobuyuki Terada,[2] Yuji Takauchi,[3] Fumitaka Saji,[4] Yasuko Nishizawa,[2] Satonori Tanaka[5] and Hiroaki Kosaka[5]**

*[1]Department of Obstetrics and Gynecology, Nishinomiya Municipal Central Hospital, Hyogo 663, [2]Department of Pathology and [3]Department of Anesthesiology, The Center for Adult Diseases, Osaka 537 and Departments of [4]Obstetrics and Gynecology and [5]Physiology, Osaka University Medical School, Suita, Osaka 565, Japan*

**We investigated the cytotoxic effects of various cytokines secreted by macrophages or T lymphocytes on luteal cells, and the role of nitric oxide (NO) produced by luteal cells in cytotoxic actions of cytokines. Mouse luteal cells were cultured in serum-free medium with interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) alone, or with various combinations of these cytokines for 6 days. Cytotoxic actions of cytokines and NO production by luteal cells were evaluated by number of viable cells and the amount of nitrite and nitrate (stable metabolites of NO) in medium, respectively. IFN-γ (1000 U/ml), TNF-α (3000 U/ml), or IL-1β (30 U/ml) alone, and the combination of TFN-α and IL-1β (10 U/ml) did not decrease number of viable cells and was without effects on NO production. The combination of IFN-γ and IL-1β (10 U/ml) also did not decrease the number of viable cells, while it increased NO production a little but significantly. Combinations of INF-γ and TNF-α, and IFN-γ, TNF-α and IL-1β (10 U/ml) markedly decreased number of viable cells. The combination of IFN-γ and TNF-α increased NO production a little but significantly, and the combination of three cytokines (IFN-γ, TNF-α, and IL-1β) caused a greater increase in NO production. An NO synthase inhibitor, L-$N^G$-monomethy-L-arginine (0.5 mM) or aminoguanidine (0.5 mM) abolished increases in NO production induced by combinations of IFN-γ and TNF-α, and IFN-γ, TNF-α and IL-1β completely without effects on number of viable cells. The present results indicate that combinations of cytokines including IFN-γ and TNF-α induce death of cultured mouse luteal cells, and that the cytotoxic actions of these cytokines are independent of NO production by luteal cells.**

*J. Steroid Biochem. Molec. Biol.*, Vol. 55, No. 3/4, pp. 291–296, 1995

## INTRODUCTION

Macrophages and T lymphocytes are found in the regressing corpus luteum, and have been suggested to play a role in the regression of corpus luteum [1]. Recently, Benyo and Pate [2] and ourselves [3] have shown that tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which are secreted by macrophages and T lymphocytes, respectively, induce death of luteal cells synergistically, and have suggested that these cells participate in structural luteolysis (destruction and removal of luteal cells) through the secretion of cytokines. However, the mechanisms by which TNF-α and IFN-γ exert the cytotoxic effect on luteal cells are not known.

Nitric oxide (NO) is a multifunctional molecule produced by the NO synthase (NOS) from L-arginine [4, 5]. Two types of NOS have been identified [4, 5]. Constitutively expressed NOS (constitutive NOS) is calcium-dependent. It is notably expressed in the endothelial cells and some neurons, and mediates vasodilation and neurotransmission. The other type of NOS, inducible NOS (iNOS), is calcium-independent, and is found in a variety of tissues. The increase in its activity under a variety of stimuli requires protein synthesis.

Several studies have shown that a variety of cytokines induce an increase in the iNOS activity in various cells

*Correspondence to T. Jo at: Department of Obstetrics and Gynecology, Nishinomiya Municipal Central Hospital, 8-24 Hayashida, Nishinomiya, Hyogo 663, Japan.
Received 29 May 1995; accepted 3 Aug. 1995.

and tissues [6–15] and that NO exerts cytotoxic effects [12–19]. Therefore, to elucidate the role of NO in death of luteal cells induced by cytokines secreted by macrophages and T lymphocytes, we investigated correlation between cytotoxic effects of these cytokines and NO production of luteal cells in culture of mouse luteal cells. Since macrophages secrete interleukin-1 (IL-1) as well as TNF-$\alpha$, we also examined the cytotoxic effects of IL-1$\beta$ alone and in combination with TNF-$\alpha$ and/or IFN-$\gamma$.

## MATERIALS AND METHODS

### *Materials*

Type I collagen coated plates with 6 or 12 wells were purchased from Iwaki Glass Co. (Chiyoda, Tokyo, Japan). Chemicals were obtained from the following sources: Ham's F12/Dulbecco's Modified Eagle's Medium (DMEM) [1:1 mixture] without phenol red, trypsin-EDTA solution (0.5% trypsin, 5.3 mM EDTA) and TRIzol Reagent from GIBCO Laboratories (Grand Island, NY, U.S.A.); collagenase, nicotinamide, $\beta$-NAD, Nitro Blue Tetrazolium, and dehydroepiandrosterone from Wako Pure Chemicals Industries (Chyuo, Osaka, Japan); gentamicin, insulin from bovine pancreas, deoxyribonuclease 1 (DNase 1), human transferrin, $N^G$-monomethyl-L-arginine (L-NMMA) and aminoguanidine from Sigma Chemical Co. (St Louis, MO, U.S.A.); reverse transcriptase–polymerase chain reaction (RT-PCR) kit from Takara Co. (Chyuo, Kyoto, Japan). Recombinant mouse interferon-$\gamma$ (IFN-$\gamma$) and recombinant human tumor necrosis factor-$\alpha$ (TNF-$\alpha$) were supplied by Shionogi Pharmaceutical Co. (Chyuo, Osaka, Japan) and Dainippon Pharmaceutical Co. (Chyuo, Osaka, Japan), respectively.

### *Mice*

2-month-old BALB/c mice were purchased from SLC (Hamamatu, Shizuoka, Japan). Female and male mice were kept together overnight, and the following morning female mice were examined for vaginal plugs (day 0 of pregnancy). Females were killed by dislocation on days 10–15 of pregnancy for collection of corpora lutea. All mice were kept at 25°C under controlled lighting conditions (12 h light/12 h darkness) and allowed free access to water and pellet food.

### *Luteal cell culture*

Both ovaries were removed from mice on days 10–15 of pregnancy and corpora lutea were separated from these ovaries under a dissection microscope. The corpora lutea were cut into pieces, and incubated in Hank's balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$ containing collagenase (2 mg/ml) for 120 min at 37°C with repeated pipetting at 10 min intervals. Dispersed luteal cells were filtered through a cell stainer with a 70 $\mu$m mesh nylon screen (Beckton Dickinson; Franklin Lakes, NJ, U.S.A.) and collected by centrifugation at 500 $g$ for 10 min. About $2 \times 10^6$ luteal cells were obtained from 20 mice. The viability of these luteal cells in all preparations of dispersed luteal cells, estimated by the trypan blue exclusion test, was more than 92%. Luteal cells seeded at a density of $2.0 \times 10^5$ and $4.0 \times 10^5$ viable cells/well into 12- and 6-multiwell collagen coated plates, respectively, were cultured in Ham's F12/DMEM medium without phenol red supplemented with insulin (10 $\mu$g/ml), gentamicin (20 $\mu$g/ml) and transferrin (10 $\mu$g/ml) for 24 h. Then the culture medium was removed, and the cells were cultured in medium containing cytokine(s) in the presence or absence of a NOS inhibitor. The day of start of culture under various conditions was designated as day 0 of culture. The volumes of culture medium in 12- and 6-multiwell plates were 2.0 and 4.0 ml/well, respectively, and the medium was changed on day 3.

### *Histochemistry of 3$\beta$-hydroxysteroid dehydrogenase*

Luteal cells were seeded in 12-multiwell plates at a density of $2 \times 10^5$ viable cells/well. After 24 h, attached cells in 5 wells were separately collected after treatment with trypsin–EDTA solution, and were smeared on slideglasses and air-dried. Histochemistry of 3$\beta$-hydroxysteroid dehydrogenase was carried out by a modification of the method described by Wattenberg [20]. The reaction mixture for the histochemistry was made by mixing 4 ml of propylene glycol containing dehydroepiandrosterone (1.6 mg) and 52 ml of 0.1 M phosphate buffer (pH 7.4) containing nicotinamide (9 mg), $\beta$-NAD (19.2 mg) and Nitro Blue Tetrazolium (8 mg). Slideglasses were incubated in the reaction mixture at 37°C for 100 min. About 500 cells on each slideglass were examined, and the percentage of stained cells was determined.

### *Cell attachment and growth*

Luteal cells were seeded in 12-multiwell plates at a density of $2 \times 10^5$ viable cells/well. After 24 h, attached cells in 5 wells were separately collected after treatment with trypsin–EDTA solution, and the number of viable attached cells in each well was determined. Media in the other wells were changed, and then the luteal cells were cultured in medium with or without TNF-$\alpha$ (3000 U/ml) and IFN-$\gamma$ (1000 U/ml) for 3 or 6 days with a medium change on day 3. In each culture, 5 or 6 wells were used. After culture, attached cells in each well were collected, and the number of viable cells in each well was determined.

### *Effects of cytokines on NO production and the number of viable cells*

Luteal cells were seeded in 12-multiwell plates at a density of $2 \times 10^5$ viable cells/well. After 24 h medium was changed and then these cells were cultured in medium containing cytokine(s) in the presence or absence of a NOS inhibitor for 6 days. In each culture

condition, 4 or 5 wells were used. Cultures without cytokines and NOS inhibitors served as controls. On days 3 and 6, culture medium in each well was collected, and centrifuged at 1000 *g* for 10 min, and the supernatant was stored at −20°C until assay for nitrite and nitrate. On day 6, attached cells in each well were collected and the number of viable cells in each well was determined.

*Number of viable cells*

Cells were suspended in 0.1 ml of Dulbecco's phosphate buffered saline, pH 7.2 (PBS), and then mixed with 0.1 ml of 0.3% trypan blue in PBS. The number of viable cells was counted in a hemocytometer.

*Assay of nitrite and nitrate*

The amount of nitrite and nitrate was measured by an automated procedure based on the Griess reaction. Briefly, samples were passed through a cadmium column to reduce nitrate to nitrite. Then nitrite was treated with the Griess reagents to form a chromophore with absorption at 540 nm. The amount of nitrite and nitrate produced by cultured cells was calculated by subtracting the amount of nitrite and nitrate in medium before culture from that in medium after culture [21].

*RT–PCR of iNOS mRNA*

Luteal cells were seeded in 6-multiwell plates at a density of $4 \times 10^5$ cells/well. After 24 h, medium was changed, and then these cells were cultured in medium without cytokines, with IFN-$\gamma$ (1000 U/ml) and TNF-$\alpha$ (3000 U/ml), or with IFN-$\gamma$ (1000 U/ml), TNF-$\alpha$ (3000 U/ml) and IL-1$\beta$ (10 U/ml) for 3 days. After culture, total RNA in attached cells was extracted with the TRIzol reagent (GIBCO Laboratories) according to the manufacturer's instruction. Conversion of RNA to cDNA and amplification of iNOS cDNA or $\beta$-actin cDNA were carried out using a Takara RT-RCR kit. The solution (20 $\mu$l) for production of cDNAs consisted of 1 $\mu$g RNA, 5 mM $MgCl_2$, 1 mM dNTPs, 1 U/ml RNase inhibitor, 0.25 U/ml AMV reverse transcriptase, and 2.5 $\mu$M oligo $(dT)_{20}$ primer in RNA PCR buffer (20 mM Tris–HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.01% Triton X-100, 50% glycerol, pH 7.5). This solution was subsequently incubated at 45°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min. Then, the solution (20 $\mu$l) was mixed with a mixture (80 $\mu$l) that consisted of 4 mM $MgCl_2$, 2.5 U/100 $\mu$l Takara Taq polymerase, 0.2 $\mu$M upstream and downstream oligonucleotide primers in RNA PCR buffer. Upstream and downstream primers for iNOS cDNA and $\beta$-actin cDNA were 5′-CCACCTTGTTCAGCTACGCC-3′ and 5′-GGACATCAAAGGTCTCACAG-3′, and 5′-TAAAGACCTCTATGCCAACAC-3′ and 5′-CTCCTGCTGATCCACAT-3′, respectively [12, 22]. cDNA of iNOS or $\beta$-actin was amplified by 25 thermal cycles, that is 30 s at 95°C, 30 s at 55°C and 30 s at 72°C. RCR products of iNOS cDNA from 2 $\mu$g RNA, and those of $\beta$-actin from 0.1 $\mu$g RNA were electrophoresed on 3% agarose gel containing 0.1 $\mu$g/ml ethidium bromide.

*Statistics*

Statistical significance ($P < 0.05$) was determined by the Mann–Whitney test.

## RESULTS

*Histochemistry of 3$\beta$-hydroxysteroid dehydrogenase*

Luteal cells ($2 \times 10^5$ viable cells/well) were cultured for 24 h for cell attachment, and attached cells harvested from each well were stained by histochemistry of 3$\beta$-hydroxysteroid dehydrogenase. The percentage (mean ± SE: $n = 5$) of stained cells was 96.6 ± 1.2%.

*Cell attachment and growth*

Luteal cells ($2 \times 10^5$ viable cells/well) were cultured for 24 h for cell attachment, and then attached cells were cultured in medium with or without TNF-$\alpha$ (3000 U/ml) and IFN-$\gamma$ (1000 U/ml) for 3 or 6 days. On day 0 of culture (day of the start of culture with or without cytokines), the number of viable attached cells was $4.74 \pm 1.1 \times 10^4$ cells/well (mean ± SE: $n = 5$) and 23.7 ± 5.5% of the seeded cells were attached to the wells. The numbers of viable cells (means ± SE: $n = 5$) on days 3 and 6 of culture without cytokines were $9.5 \pm 1.5 \times 10^4$ and $10.7 \pm 0.8 \times 10^4$ cells/well, respectively; the number of viable cells increased about 2-fold on day 3, but there was no further increase on day 6. The numbers of viable cells (means ± SE: $n = 6$) on days 3 and 6 of culture with TNF-$\alpha$ and IFN-$\gamma$ were $9.0 \pm 0.7 \times 10^4$ and $3.6 \pm 0.3 \times 10^4$ cells/well, respectively. The number of viable cells on day 3 of culture with TNF-$\alpha$ and IFN-$\gamma$ was similar to that in culture without cytokines, but on day 6, it decreased to about one-third of the cell number on day 3.

*Effects of cytokine(s) on NO production and number of viable luteal cells*

Table 1 shows the effects of a cytokine or a NOS inhibitor on NO production and number of viable luteal cells. A cytokine, IL-1$\beta$ (30 U/ml), IFN-$\gamma$ (1000 U/ml) or TNF-$\alpha$ (3000 U/ml), and a NOS inhibitor, L-NMMA (0.5 mM), or aminoguanidine (0.5 mM) exerted no significant effects on NO production and number of viable cells.

Table 2 shows the effects of various combinations of cytokines on NO production and number of viable luteal cells. The combination of TNF-$\alpha$ (3000 U/ml) and IL-1$\beta$ (10 U/ml) affected neither NO production nor number of viable cells. Combinations of IFN-$\gamma$ (1000 U/ml) and IL-1$\beta$ (10 U/ml), and IFN-$\gamma$ (1000 U/ml) and TNF-$\alpha$ (3000 U/ml) increased NO production during days 0–3, and days 4–6 a little, but significantly. The combination of IFN-$\gamma$ and TNF-$\alpha$ decreased the number of viable cells by about 40%, but

*Table 1. Effects of various cytokines on NO production and number of viable cells*

| Treatment | Nitrite + Nitrate (μM) Days 0–3 | Days 4–6 | No. of viable cells |
|---|---|---|---|
| None (control) | 2.7 ± 0.3 | 1.4 ± 0.3 | 100.0 ± 7.5 |
| IL-1β (30 U/ml) | 1.7 ± 0.2 | 1.7 ± 0.5 | 103.5 ± 16.6 |
| IFN-γ (1000 U/ml) | 3.4 ± 0.4 | 2.3 ± 0.1 | 126.2 ± 10.9 |
| TNF-α (3000 U/ml) | 2.2 ± 0.2 | 1.5 ± 0.1 | 96.9 ± 17.4 |
| L-NMMA (0.5 mM) | 2.0 ± 0.2 | 1.0 ± 0.1 | 106.2 ± 9.8 |
| Aminoguanidine (0.5 mM) | 2.5 ± 0.1 | 1.4 ± 0.1 | 98.8 ± 9.7 |

Luteal cells were cultured in medium containing a cytokine or a NOS inhibitor for 6 days. On days 3 and 6, medium was collected, and the amount of nitrite and nitrate in the medium was measured. On day 6, the number of viable cells was determined. The mean number ($8.7 \pm 0.6 \times 10^4$) of viable cells in the control culture was regarded as 100.0. Results represent means ± SE for 4 or 5 wells.

that of IFN-γ and IL-1β did not. The combination of three cytokines increased NO production during days 0–3, and days 4–6 markedly, and decreased the number of viable cells by about 50%. A NOS inhibitor, L-NMMA (0.5 mM) abolished the increase in NO production induced by the combination of three cytokines completely, but did not affect the number of viable cells.

Table 3 shows the effects of combinations of IFN-γ (1000 U/ml) and TNF-α at various concentrations on NO production and the number of viable cells. Only the combination of IFN-γ and TNF-α at 3000 U/ml increased NO production during days 0–3, but all combinations of IFN-γ and TNF-α at 500, 1000, and 3000 U/ml increased NO production during days 4–6. Combinations of IFN-γ and TNF-α at all concentrations decreased the number of viable cells, the decrease being dependent on concentrations of TNF-α. NOS inhibitors, L-NMMA (0.5 mM) and aminoguanidine (0.5 mM) completely inhibited NO production induced by the combination of IFN-γ and TNF-α (3000 U/ml), but did not affect the number of viable cells.

*Table 2. Effects of various combinations of cytokines on NO production and number of viable cells*

| Treatment | Nitrite + Nitrate (μM) Days 0–3 | Days 4–6 | No. of viable cells |
|---|---|---|---|
| None (control) | 0.9 ± 0.1 | 1.8 ± 0.1 | 100.0 ± 12.1 |
| TNF-α + IL-1β | 0.8 ± 0.1 | 2.2 ± 0.4 | 98.6 ± 9.4 |
| IL-1β + IFN-γ | 1.5 ± 0.1* | 3.3 ± 0.2* | 105.6 ± 6.0 |
| TNF-α + IFN-γ | 1.6 ± 0.1* | 5.4 ± 0.3* | 61.3 ± 2.2* |
| TNF-α + IFN-γ + IL-1β | 6.5 ± 0.2* | 14.9 ± 0.5* | 51.8 ± 2.2* |
| TNF-α + IFN-γ + IL-1β + L-NMMA | 1.0 ± 0.2† | 1.0 ± 0.1† | 48.7 ± 5.3* |

Luteal cells were cultured in medium containing various combinations of cytokine in the presence or absence of a NOS inhibitor, L-NMMA. Concentrations of TNF-α, IL-1β, IFN-γ and L-NMMA were 3000, 10, 1000 U/ml and 0.5 mM, respectively. On days 3 and 6, medium was collected, and the amount of nitrite and nitrate in the medium was measured. On day 6, the number of viable cells was determined. The mean number ($12.2 \pm 1.5 \times 10^4$) of viable cells in the control culture was regarded as 100.0. Results represent means ± SE for 4 or 5 wells.

*$P < 0.05$, significant difference from the value of the control culture.

†$P < 0.05$, significant difference from the value of culture with TNF-α, IFN-γ and IL-1β.

*Table 3. Effects of combinations of IFN-γ and TNF-α at various concentrations on NO production and number of viable cells*

| Treatment | Nitrite + Nitrate (μM) Days 0–3 | Days 4–6 | No. of viable cells |
|---|---|---|---|
| None (control) | 0.9 ± 0.1 | 0.9 ± 0.1 | 100.0 ± 4.8 |
| TNF-α (500 U/ml) + IFN-γ | 1.2 ± 0.1 | 2.9 ± 0.1* | 69.3 ± 4.2* |
| TNF-α (1000 U/ml) + IFN-γ | 1.3 ± 0.2 | 3.5 ± 0.2* | 60.2 ± 12.0* |
| TNF-α (3000 U/ml) + IFN-γ | 2.4 ± 0.8* | 3.3 ± 0.2* | 32.2 ± 4.9* |
| TNF-α (3000 U/ml) + IFN-γ + L-NMMA | 1.0 ± 0.2† | 1.4 ± 0.2† | 30.3 ± 1.7* |
| TNF-α (3000 U/ml) + IFN-γ + aminoguanidine | 1.1 ± 0.1† | 1.2 ± 0.1† | 32.3 ± 1.1* |

Luteal cells seeded at a density of $2 \times 10^5$ vaible cells/well were cultured in meium with INF-γ (1000 U/ml) and TNF-α at various concentrations in the presence or absence of a NOS inhibitor, L-NMMA (0.5 mM) or aminoguanidine (0.5 mM) for 6 days. On days 3 and 6, medium was collected, and the amount of nitrite and nitrate was measured. On day 6, the number of viable cells was determined. The mean number ($10.4 \pm 5.0 \times 10^4$) of viable cells in the control culture was regarded as 100.0. Results represent means ± SE for 4 or 5 wells.

*$P < 0.05$, significant difference from the value of the control culture.

†$P < 0.05$, significant difference from the value of culture with IFN-γ and TNF-α (3000 U/ml).

*Effects of cytokines on iNOS mRNA expression*

Luteal cells were cultured in medium without cytokines, or with IFN-γ (1000 U/ml) and TNF-α (3000 U/ml), or IFN-γ (1000 U/ml), TNF-α (3000 U/ml) and IL-1β (10 U/ml) for 3 days, and expression of iNOS mRNA in cultured luteal cells was examined by a RT–PCR method (Fig. 1). The

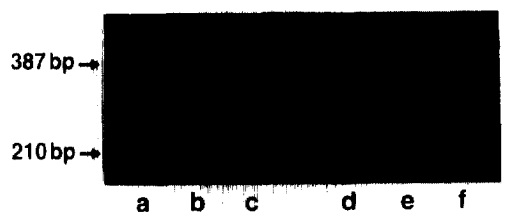


**Fig. 1. Expression of iNOS mRNA in mouse luteal cells cultured in medium with or without cytokines for 3 days. Ethidium bromide-stained agarose gel of 387 bp PCR products of iNOS cDNA (a, b, c) and 210 bp sequence of β-actin (d, e, f) is shown. (a and d) Culture with IFN-γ (1000 U/ml), TNF-α (3000 U/ml) and IL-1β (10 U/ml), (b and e) culture with IFN-γ (1000 U/ml) and TNF-α (3000 U/ml), (c and f) culture without cytokines.**

iNOS mRNA expression was detected in luteal cells cultured in medium with IFN-$\gamma$, TNF-$\alpha$ and IL-1$\beta$, but not in those cultured in medium with IFN-$\gamma$ and TNF-$\alpha$ or without cytokines.

## DISCUSSION

In our luteal cell culture system, dispersed cells from corpora lutea were used. These cells contain various cell types, such as endothelial cells, fibroblasts and blood cells besides luteal cells [23]. However, after 24 h of culture in serum-free medium for cell attachment, almost all attached cells were stained by histochemistry of 3$\beta$-hydroxysteroid dehydrogenase, suggesting that these cells are luteal cells. Therefore, the effects of cytokines and/or NOS inhibitors found in our culture system seem to reflect their effects on luteal cells.

Combinations of cytokines including both IFN-$\gamma$ and TNF-$\alpha$ decreased the number of viable cells on day 6 of culture. In culture without cytokines, the number of viable cells increased about 2-fold on day 3, and remained unchanged on day 6. The combination of IFN-$\gamma$ and TNF-$\alpha$ did not affect the number of viable cells on day 3, but decreased it on day 6. Therefore, the decrease in cell number on day 6 caused by these two cytokines is not due to their inhibitory action on cell growth, but to their cytotoxic action on cells during days 4–6. In addition, a cytokine alone did not decrease the number of viable cells, and synergism of IFN-$\gamma$ and TNF-$\alpha$ was required for their cytotoxic action as reported previously [3].

A cytokine alone did not induce NO production, but NO production was enhanced by synergistic actions of cytokines. Combinations of IFN-$\gamma$ and TNF-$\alpha$, and IFN-$\gamma$, TNF-$\alpha$ and IL-1$\beta$ caused an increase in NO production as well as a decrease in cell number. However, a NOS inhibitor completely abolished the increase in NO production without effects on cell number. Therefore, cytotoxic actions of combinations of cytokines are independent of NO production induced by them. The result that the combination of IFN-$\gamma$ and IL-1$\beta$ increased NO production without effects on cell number is consistent with this indication. In cultures of various cells or tissues, some studies suggested that cytotoxity induced by cytokines is dependent on NO production stimulated by cytokines, and the other studies did not. In culture of rat ovarian cells without luteal cells, Ellman *et al.* [13] reported that NO mediated IL-1$\beta$-induced cellular cytotoxity, but in contrast, Ben-Shlomo *et al.* [10] reported that IL-1$\beta$-induced cellular cytotoxity was independent of NO. The reason for the different results between the two studies is unknown. In culture of transformed murine fibroblasts, rat insulinoma cells, dispersed islet cells of mice, and mouse pancreatic islets, NO is shown to be a mediator of cytotoxic actions of cytokines [12–15], but in culture of human pancreatic islets, cytotoxity caused by cytokines is shown to be independent of NO [11]. Cui *et al.* [24] reported that sensitivities of cells to cytotoxic actions of NO were different between two types of tumor cells. Thus, contributions of NO in cytotoxic actions of cytokines may differ depending on sensitivities of various types of cells to NO or levels of NO produced by these cells.

The expression of iNOS mRNA was detected in luteal cells cultured in medium with IFN-$\gamma$, TNF-$\alpha$ and IL-1$\beta$, although it was not detected in luteal cells cultured in medium with IFN-$\gamma$ and TNF-$\alpha$, and without cytokines. These results indicate that iNOS is responsible for the increase in NO production induced by the combination of IFN-$\gamma$, TNF-$\alpha$ and IL-1$\beta$. Non-detection of iNOS mRNA expression in luteal cells cultured in medium with IFN-$\gamma$ and TNF-$\alpha$ seems to be ascribed to the smaller increase in NO production induced by the combination of IFN-$\gamma$ and TNF-$\alpha$, compared to that induced by the combination of three cytokines.

NO exerts many of its actions by binding iron-containing enzymes. NO is known to enhance the activity of the heme containing enzyme, cyclooxygenase, leading to stimulation of prostaglandin production [25]. Benyo and Pate [2] showed that IFN-$\gamma$ and TNF-$\alpha$ synergistically increased the prostaglandin (PG) $F_{2\alpha}$ production in cultured bovine luteal cells, and Nothnick and Pate [26], and Townson and Pate [27] reported that IL-1$\beta$ also enhanced $PGF_{2\alpha}$ in cultured bovine luteal cells. In our study, the combination of IFN-$\gamma$ and TNF-$\alpha$ increased NO production by cultured mouse luteal cells, but IL-1$\beta$ did not. Furthermore, Ben-Shlomo *et al.* [10] showed that IL-1$\beta$-stimulated PG $E_2$ production by cells dispersed from rat ovaries without corpora lutea was independent of IL-1$\beta$-induced NO production. Therefore, it is likely that cytokine-induced production of prostaglandins by luteal cells is independent of NO produced by these cells.

The cytochrome *P*450 steroidogenic enzymes are also iron containing enzymes [28] so it is possible that NO modulates activities of these enzymes. Van Voorhis *et al.* [29] reported that NO donors added to culture medium decreased progesterone and estradiol secretion by human granulosa-luteal cells dose-dependently. On the other hand, Benyo and Pate [2] showed that IFN-$\gamma$ and TNF-$\alpha$ synergistically decreased LH-stimulated progesterone secretion by cultured bovine luteal cells. In our study, IFN-$\gamma$ and TNF-$\alpha$ synergistically increased NO production by mouse luteal cells. Thus, it is possible that NO produced by luteal cells through synergistic actions of cytokines plays a role in the decrease in progesterone secretion by these cells. This possibility may be worth further investigation.

In conclusion, the present results indicate that death of cultured luteal cells induced by synergistic actions of cytokines is independent of NO produced by these cells.

*Acknowledgement*—This work was supported in part by a grant from the Osaka Cancer Foundation.

## REFERENCES


1. Bagavandoss P., Wiggins R. C., Kunkel S. L., Remic D. G. and Keyes R. L.: Tumor necrosis factor production and pseudopregnancy and pregnancy in rabbits. *Biol. Reprod.* **42** (1990) 367–376.
2. Benyo D. F. and Pate J. L.: Tumor necrosis factor-$\alpha$ alters bovine luteal cell synthetic capacity and viability. *Endocrinology* **130** (1992) 854–860.
3. Jo T., Tomiyama T., Ohashi K., Saji F., Tanizawa O., Nisizawa Y. and Terada N.: Apoptosis of cultured mouse luteal cells induced by tumor necrosis factor-$\alpha$ and interferon-$\gamma$. *Anat. Rec.* **241** (1995) 70–76.
4. Moncada S. R., Palmer M. J. and Higgs E. A.: Nitric oxide: physiology, pathophysiology. *Pharmac. Rev.* **43** (1992) 109–142.
5. Nathan C.: Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6** (1992) 3051–3064.
6. Spitzer J. A.: Cytokine stimulation of nitric oxide formation and differential regulation in hepatocytes and nonparenchymal cells of endotoxemic rats. *Hepatology* **19** (1994) 217–228.
7. Damoulis P. D. and Hauschka P. V.: Cytokines induce nitric oxide production in mouse osteoblasts. *Biochem. Biophys. Res. Commun.* **201** (1994) 924–931.
8. Feinstein D. L., Galea E., Cermak J., Chugh P., Lyandvert L. and Reis D. J.: Nitric oxide synthase expression in glial cells: supression by tyrosine kinase inhibitors. *J. Neurochem.* **62** (1994) 811–814.
9. Ben-Shlomo I., Kokia E., Jackson M. J., Adashi E. Y. and Payne D. W.: Interleukin-1$\beta$ stimulates nitric production in the rat ovary: Evidence for heterogous cell–cell interaction and for insulin-mediated regulation of the inducible isoform of nitric oxide synthase. *Biol. Reprod.* **51** (1994) 310–318.
10. Ben-Shlomo I., Adashi E. Y. and Payne D. W.: The morphologic/ cytotoxic and prostaglandin-stimulating activities of IL-1$\beta$ in the rat ovary are nitric oxide independent. *J. Clin. Invest.* **94** (1994) 1463–1469.
11. Ravinovitch A., Suare-Pinzon W. L., Strynadka K., Schulz R., Lakey J. R., Warnock G. L. and Rajotte R. V.: Human pancreatic islet $\beta$-cell destruction by cytokine is independent of nitric oxide production. *J. Clin. Endocr. Metab.* **79** (1994) 1058–1062.
12. Yamada K., Otabe S., Inada C., Takane N. and Nonaka K.: Nitric oxide and nitric oxide synthase mRNA induction in mouse islet cells by interferon-$\gamma$ plus tumor necrosis factor-$\alpha$. *Biochem. Biophys. Res. Commun.* **197** (1993) 22–27.
13. Ellman C., Corbett J. A., Misko T. P., McDaniel M. and Beckerman K. P.: Nitric oxide mediates interleukin-1-induced cytotoxity in the rat ovary. *J. Clin. Invest.* **92** (1993) 3053–3056.
14. Xie K., Huang S., Dong Z. and Fidler I. J.: Cytokine-induced apoptosis in transformed murine fibroblasts involves synthesis of endogenous nitric oxide. *Int. J. Oncol.* **3** (1993) 1043–1048.
15. Cetkovic-Cvrlje M. and Eizirik D. L.: TNF-$\alpha$ and IFN-$\gamma$ potentiate the deleterious effects on IL-1$\beta$ on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* **19** (1994) 217–228.
16. Sarih M., Souvannavong V. and Adam A.: Nitric oxide induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* **191** (1993) 503–508.
17. Stuehr D. J. and Nahan C. F.: Nitric oxide, a macrophage product responsible for cytostasia and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169** (1989) 1543–1555.
18. Hibbs Jr J. B.: Nitric oxide: cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157** (1988) 87–94.
19. Nguyen T. D., Branson C. L., Crespi B. W., Wishnok J. S. and Tannenbaum S. R.: DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 3930–3934.
20. Wattenberg L. W.: Microscopic histochemical demonstration of steroid-3$\beta$-ol-dehydrogenase in tissue sections. *J. Histochem. Cytochem.* **6** (1958) 225–232.
21. Kosaka H., Sakaguchi H., Sawai Y., Kumura E., Seiyama A., Chen S. and Shiga T.: Effect of interferon-$\gamma$ on oxide hemoglobin production in endotoxin-treated rats and its synergism with interleukin-1 or tumor necrosis factor. *Life Sci.* **54** (1994) 1523–1529.
22. Tokunaga K., Taniguchi H., Yoda K., Shimizu M. and Sakiyama S.: Nucleotide sequence of a full-length cDNA for mouse skeletal $\beta$-actin mRNA. *Nucl. Acids Res.* **14** (1986) 2892.
23. O'Shea J. D., Rodger R. J. and D'Occhio M. J.: Cellular composition of the cyclic corpus luteum of the cow. *J. Reprod. Fertil.* **85** (1989) 483–487.
24. Cui S., Reichner J. S., Mateo R. B. and Albina J. E.: Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res.* **54** (1994) 2462–2467.
25. Salvemini D., Misko T. P., Masferrer J. L., Siebert K. and Currie M. G.: Nitric oxide activates cyclooxygenase enzymes. *Proc. Natn. Acad. Sci. U.S.A.* **90** (1993) 7240–7244.
26. Nothnick W. B. and Pate J. L.: Interleukin-1 beta is a potent stimulator of prostaglandin synthesis in bovine luteal cells. *Biol. Reprod.* **43** (1990) 898–903.
27. Townson D. H. and Pate J. L.: Regulation of prostaglandin synthesis by interleukin-1$\beta$ in cultured bovine luteal cells. *Biol. Reprod.* **51** (1994) 480–485.
28. Guengerich F. P.: Characterization of human microsomal cytochrome *P*-450 enzymes. *A. Rev. Pharmac. Toxic.* **29** (1989) 241–264.
29. Van Voorhis B. J., Dunn M. S., Synder G. D. and Weiner C. P.: Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* **135** (1994) 1799–1806.