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Interleukin-1 as an Autocrine Stimulator in the Growth of Human Ovarian Cancer Cells

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ABSTRACT

The role of interleukin-1 (IL-1), a multifunctional cytokine which mediates important immune responses, was investigated in the growth of ovarian cancer cell lines *in vitro*. The messenger RNA for IL-1α and IL-1β was expressed in six and four ovarian cancer cell lines, respectively out of eight. Measurement of IL-1 in the eight cell lines by enzyme-linked immunosorbent assay revealed that two lines, MCAS and TYK-nu, secreted a high amount of IL-1α, but that none secreted IL-1β after 72 hours of incubation. The growth of these cells was significantly stimulated by the addition of recombinant IL-1α (rIL-1α) in a concentration-dependent manner in a 96 hour culture. The maximum response was obtained with 10 ng/ml of IL-1α by counting the viable cell number using trypan blue. [^{3}H]-thymidine incorporation by these cells was also stimulated by a 72 hour incubation with rIL-1α. The spontaneous growth of these cells was inhibited by the addition of anti-IL-1α antibody, anti-IL-1 receptor antibody or IL-1 receptor antagonist. These cells expressed two classes of IL-1-binding receptors on their surface as detected by [^{125}I]-labeled rIL-1α. These results indicate that IL-1α is an autocrine growth stimulator for some ovarian cancer cells and suggest that IL-1α plays an important role in the progression of this disease.

Key words: IL-1, Autocrine stimulation, Ovarian cancer cells, EGF receptor

Ovarian cancer is responsible for the highest rate of death of all gynecological malignancies. Frequently, at the time of diagnosis patients present with a disease that has spread beyond the pelvis and into the peritoneal cavity. Disappointingly, cytoreductive surgery and combination chemotherapy cannot improve their poor prognosis. Therefore, it is necessary to understand the growth mechanism of ovarian cancer cells. Tumor progression has often been attributed to oncogene overexpression or inappropriate growth factors produced by the cancer cells. In ovarian cancer, it has been reported that cytokines, including IL-1α, β, IL-6, IL-10, TNF-α, IFN-γ, M-CSF and GM-CSF are produced and their genes are expressed[1,12,16,17,23,24,30,34]. In particular, IL-1α, IL-6, TNF-α and TGF-α have been found to act as a growth factor in some ovarian cancer cells[13,14,15,29,35].

IL-1, produced not only by activated monocytes or macrophages but also by diverse cell types including NK-cells, T-cells, B-cells, endothelial cells, fibroblasts and several malignant cells, has a wide variety of biological functions for immune responses, inflammatory reactions, hematopoiesis and the endocrine system[5,6,22]. Recent studies have shown that some kinds of malignant cells produce IL-1 constitutively and that it may play a role in tumor growth. In these cells, including myeloid leukemia cells, adult T-cell leukemia cells, thyroid carcinoma cells and gastric carcinoma cells, it has been confirmed that the secretion of IL-1 and the coexpression of its receptor can lead to autocrine stimulation[3,8,20,27,39]. On the other hand, it has been reported that IL-1 itself has also a direct antiproliferative or cytocidal effect on several malignant cells[2,4,10,21].

The purpose of the current investigation was to examine the biological role of IL-1 and to understand the growth mechanism of ovarian cancer cells. In the present study we examined whether the secretion of IL-1 and the expression of its receptor were consistent properties, whether exogenous addition of IL-1 stimulated the growth of these cells, whether neutralizing antibodies against IL-1 inhibited their growth, and provided information on the role of IL-1 as an autocrine growth stimulator of ovarian cancer cells *in vitro*.

MATERIALS AND METHODS

Cell Cultures.

Two human ovarian cancer cell lines, MCAS and TYK-nu[9,37] (Health Science Research Resources Bank JCRB0240, JCRB0234.0), were

cultured in Eagle's MEM medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with L-gultamine (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) and 20% or 10% heat-inactivated fetal bovine serum (FBS, ICN Biomedicals Japan Co. Ltd., Tokyo, Japan), respectively. OVK-18 line[32] (Cancer Cell Repository, Tohoku University TKG0323) was cultured in RPMI1640 medium (Nissui Pharmaceutical Co. Ltd.) supplemented with L-glutamine (GIBCO BRL, Grand Island, NY), 10% heat-inactivated FBS and 1% penicillin-streptomycin (GIBCO). Five ovarian cancer cell lines RMG-I, RMG-II, RTSG, RUMG-S and RMUG-L[18,19,26,31] (Institute For Fermentation IFO50315, IFO50316, IFO50318, IFO5050320, IFO50319), were cultured in Ham's F-12 medium (GIBCO) supplemented with L-glutamine, 10% heat-inactivated FBS and 1% penicillin-streptomycin. All cell lines were maintained under conditions of humidified 5% CO_2 in air at 37°C. The medium was changed every 3 days and the cells were subcultured once a week. For subculture and experiments, monolayers were detached by treating with 0.25% trypsin-0.02% EDTA.

Preparation of RNA and Reverse Transcription to cDNA

Total cellular RNA was prepared from the cells using A Quick Prep Total RNA Extraction Kit (Pharmacia Biotech, Uppsala, Sweden). Subsequently, cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (SuperScript II RNase H⁻ Reverse Transcriptase; GIBCO) as recommended by the manufacturer.

RT-PCR Amplification

Thirty eight microliters of PCR mixture was added to 12 μl of first-strand 500 ng of cDNA. The PCR mixture contained 5 μl of 10×buffer [500 mM Tris·HCl/750 mM KCl/100 mM DTT/30 mM $MgCl_2$ (each; GIBCO)], 0.5 μl of dNTP (25 mM) (Pharmacia), 24.5 μl of sterile water, 1 μl of each primer (0.5 μl/ml) and 1 μl of Taq DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). The reaction mixture was amplified with a ASTEC thermal cycler (ASTEC Co. Ltd., Fukuoka, Japan) for 36 cycles. The temperature profile used was 94°C for 1 min for denaturation, 55°C for 1 min for annealing and 72°C for 1 min for primer extention. All primers were RNA specific and nonreactive with DNA. The following oligonucleotide 5' and 3' primer sequences were used: IL-1α sense: 5'-ATGGCCAAAGTTCCAGACATGTTTG-3'; IL-1α antisense: 3'-GGTTTTCCAGTATCTG-AAAGTCAGT-5'; IL-1β sense: 5'-ATGGCAGAAGTA-CCTAAGCTCGC-3'; IL-1β antisense: 3'-ACACAA-ATTGCATGGTGAAGTCAGTT-5'; β-actin sense: 5'-CCTTCCTGGGCATGGAGTCCTG-3'; β-actin antisense: 3'-GGAGCAATGATCTTGATCTTC-5'. Ethidium bromide-stained PCR products were visualized on a UV transilluminator after electrophoresis on 1.5% agarose gels.

Assay of the Production of IL-1

All the ovarian cancer cells ($2 \times 10^6 \sim 4 \times 10^6$) were cultured in dishes (Falcon 3002, Becton Dickinson Co. Ltd., Lincoln Park, NJ) and grown to subconfluence. Then the cells were rinsed once, and the media were replaced by serum-free fresh RPMI 1640 medium. The supernatant was harvested after 48 hour incubation, and immunoreactive IL-1 was measured by means of a human IL-1 enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) in duplicates as recommended by the manufacturer.

Cytokines and Cytokine Neutralizing Agents

Human recombinant IL-1α was kindly donated by Dr. M. Yamada (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan). Anti-IL-1α monoclonal antibody was purchased from Genzyme Co. Ltd. (Cambridge, MA). The IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody were kindly provided by Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan and Immunex Co. Ltd., Seattle, WA, respectively.

Cell Proliferation

Cell proliferation was measured by trypan blue dye exclusion assay and [³H]thymidine incorporation. The MCAS cells (5×10^3/well) and TYK-nu cells (1×10^4/well) in RPMI1640 medium with 1% FBS were plated in 96-well flat bottomed microtiter plates (Falcon 3072) with various concentrations of IL-1α, anti-IL-1α monoclonal antibody (anti-IL-1α MoAb), IL-1 receptor antagonist (IL-1ra) or anti-IL-1 receptor monoclonal antibody (anti-IL-1RMoAb) at 37°C for up to 96 hours in 5% CO_2 and 95% air. The growth of the cells was detected by counting the number of viable cells in the culture plates using 0.1% trypan blue in triplicate. To measure the DNA synthesis, the cells were also pulsed for the last 20 hours with 0.2 μCi of tritiated thymidine ([³H]-TdR, specific activity of 5 Ci/mM, Amersham International Plc., Amersham, Buckinghamshire, UK) and were harvested with the aid of an automated cell harvester (Abekagaku Co., Funabashi, Japan). The results were expressed as the mean±standard errors of counts per minute (cpm) of [³H]-TdR incorporated into the cells in triplicate cultures.

Detection of IL-1 Receptor and Epidermal Growth Factor Receptor (EGFR)

In [¹²⁵I]-IL-1α binding studies, The MCAS and TYK-nu cells (1×10^6) were cultured in 6-well plates and grown to confluence in RPMI1640 medium with 10% FBS. The cells were then washed

twice with RPMI1640 medium containing 1% FBS (binding buffer), and 400 μl of binding buffer containing various concentrations of [^{125}I]-IL-1α (specific activity; 2000 Ci/mmol) (1.0–40 fM) were added to each well. Parallel wells also received 4.0pM, 100-fold excess of cold IL-1α to determine nonspecific binding. After a 4 hour incubation at 4°C, the cells were washed with binding buffer and detached by treating with 0.25% trypsin–0.02% EDTA. Then radioactivity was determined in a gamma counter. The results were expressed as the mean cpm (specific binding) in which nonspecific binding was subtracted. The binding experiment of [^{125}I]-epidermal growth factor ([^{125}I]-EGF, specific activity; 750 Ci/mmol), (1–40 fM) was performed in the same way of that of [^{125}I]-IL-1α in two groups which were pretreated with 10 ng/ml IL-1α or not for 24 hours.

Statistical Analysis

All experiments were repeated at least three times and one representative result is shown as table and figure. Statistical analysis between the control group and stimulated group was performed by the Student's t test. A confidence level of <0.05 was considered significant[38].

RESULTS
IL-1 mRNA Expression of Ovarian Cancer Cells

As it is reported that several ovarian cancer cells express IL-1α and/or the β gene, we first tested the possibility that IL-1 expression was seen in ovarian cancer cell lines constitutively by RT-PCR. As shown in Fig. 1, in six and three
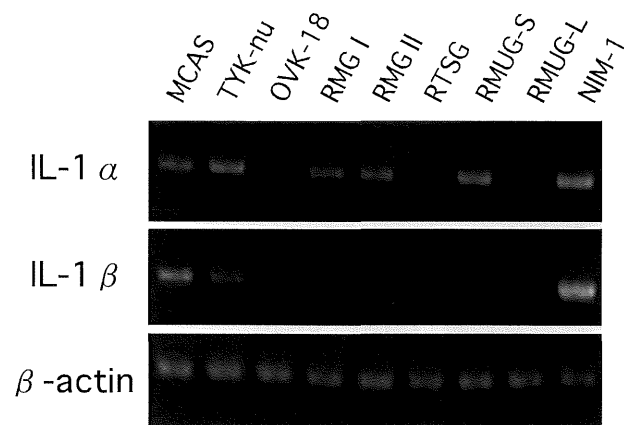

Fig. 1. Expression of IL-1α mRNA in ovarian cancer cell lines detected by RT-PCR amplification. RNA was extracted from each ovarian cancer cell and transcribed into cDNA by reverse transcription. Replicate aliquots of cDNA were amplified by PCR with gene-specific primer, and ethidium bromide stainings of PCR products were separated in agarose gel. Lane 9, NIM-1 cells, positive control. A β-actin mRNA was used as an internal control.

Table 1. Secretion of IL-1α in the supernatants of ovarian cancer cell lines

Cell lines	IL-1α (pg/10^6 cells)
MCAS	203.7 ± 6.8[b]
TYK-nu	634.4 ± 7.6
OVK-18	ND[c]
RMG-I	ND
RMG-II	30.3 ± 1.6
RTSG	ND
RMUG-S	ND
RMUG-L	ND

[a] Each subconfulent cells were cultured with serum free media for 48h. Then supernatnts were collected and the concentrations of IL-1α were assayed by ELISA.
[b] Mean ± SE
[c] not detected

cells respectively out of eight, IL-1α and β gene expression was clearly detected.

IL-1 Secretion of Ovarian Cancer Cells

To determine if the ovarian cancer cells actually secrete IL-1 protein, we examined the media of several ovarian cancer cells using an enzyme-linked immunosorbent assay which can detect the minimum amount of 7.8 pg/ml of IL-1. Two ovarian cancer cells, MCAS and TYK-nu, out of eight cells were found to secrete a high amount of IL-1α. The quantity of IL-1α secreted into the conditioned media was 203.7 ± 6.8 pg/10^6 cells in MCAS, 634.4 ± 7.6 pg/10^6 cells in TYK-nu and 30.3 ± 1.6 pg/10^6 cells in RMG-II, respectively (Table 1). IL-1β was not be detected in all the ovarian cancer cells tested although mRNA expression was observed in several cell lines (data not shown).

Growth Modulation of Ovarian Cancer Cells by Exogenous IL-1α

We next examined the effect of IL-1α on the growth of two ovarian cancer cell lines: MCAS, TYK-nu. As shown in Fig. 2, 10 ng/ml rIL-1α significantly enhanced the proliferation of these cells. In the time course of a 96 hour culture, an increase of about 25.8% in MCAS and of 29.4% in TYK-nu were observed by counting the viable cell numbers (Fig. 2). The stimulatory effect of IL-1α was in a concentration-dependent manner. The maximum stimulation of each cell was 34.2% in MCAS and 30.7% in TYK-nu in the presence of 10 ng/ml of IL-1α, respectively (Fig. 3).

rIL-1α also stimulated a concentration-dependent increase in the [^{3}H]-TdR incorporation of both cells (Fig. 4). These results indicate that IL-1α can stimulate both the cell growth and DNA synthesis of these cells.
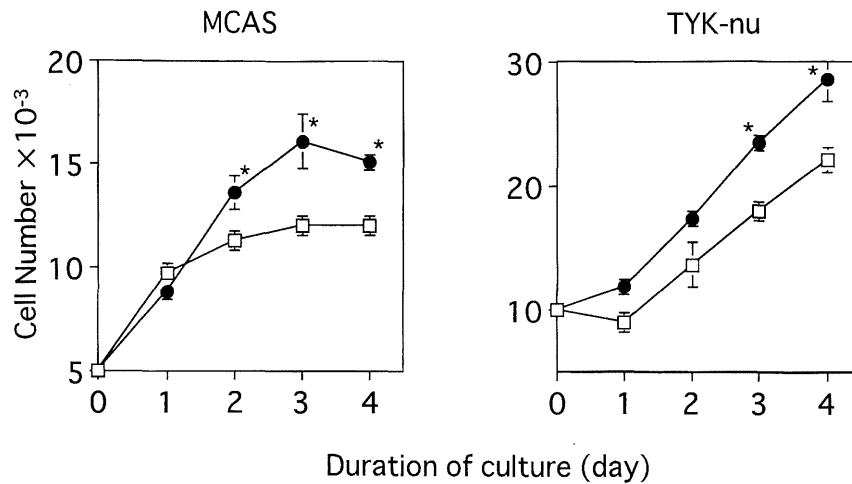
In the other six cell lines, no growth modulation

Fig. 2. Time course for stimulatory effect of exogenous IL-1α on two ovarian cancer cell lines, MCAS and TYK-nu. Cells were seeded in 96-well microtiter plates at a density of 5×10^3/well (MCAS) or 1×10^4/well (TYK-nu), cultured with 2% FBS in the presence (●) or absence (□) of 1 ng/ml IL-1α and the viable cell number was counted. Each point represents the mean ± SE of cell number in triplicatecultures; *significantly enhanced.
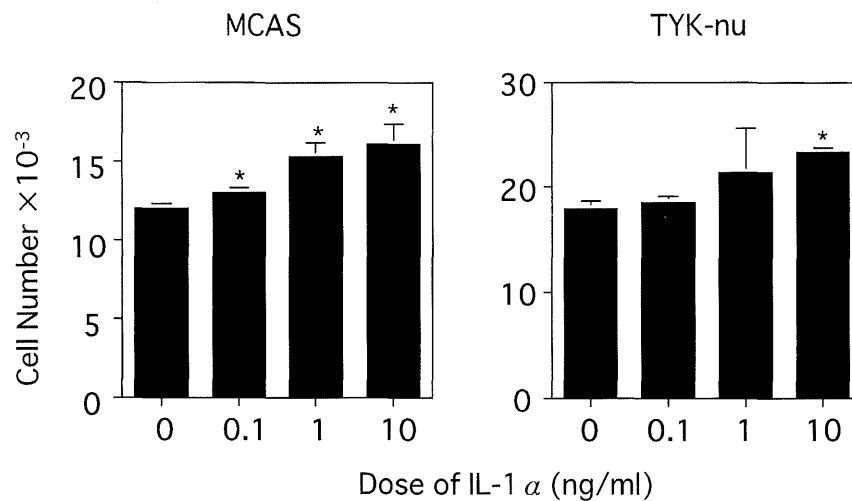

Fig. 3. Dose response of IL-1α on the growth of ovarian cancer cell lines, MCAS and TYK-nu. Cells (5×10^2 in MCAS and 1×10^3 in TYK-nu) were cultured for 72 hours with IL-1α at the concentrations indicated. Results are expressed as the mean ± SE of cell number in triplicate cultures; *significantly enhanced.

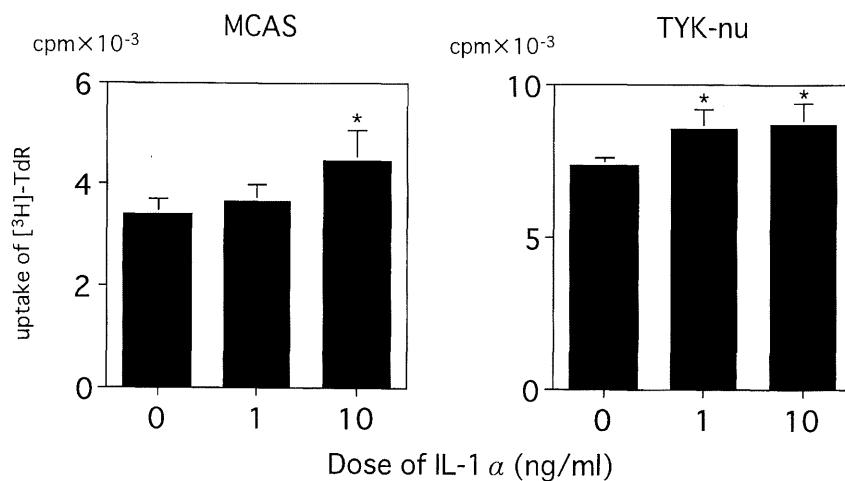**Fig. 4.** Effect of IL-1α on DNA synthesis of ovarian cancer cell lines, MCAS and TYK-nu. Cells (5×10^2 in MCAS and 1×10^3 in TYK-nu) were cultured for 72 hours with IL-1α at the concentrations indicated and labeled with [³H]-TdR for the last 20 hours. Results are expressed as cpm of [³H]-TdR uptake of tumor cells (mean ± SE of triplicate cultures); *significantly enhanced.
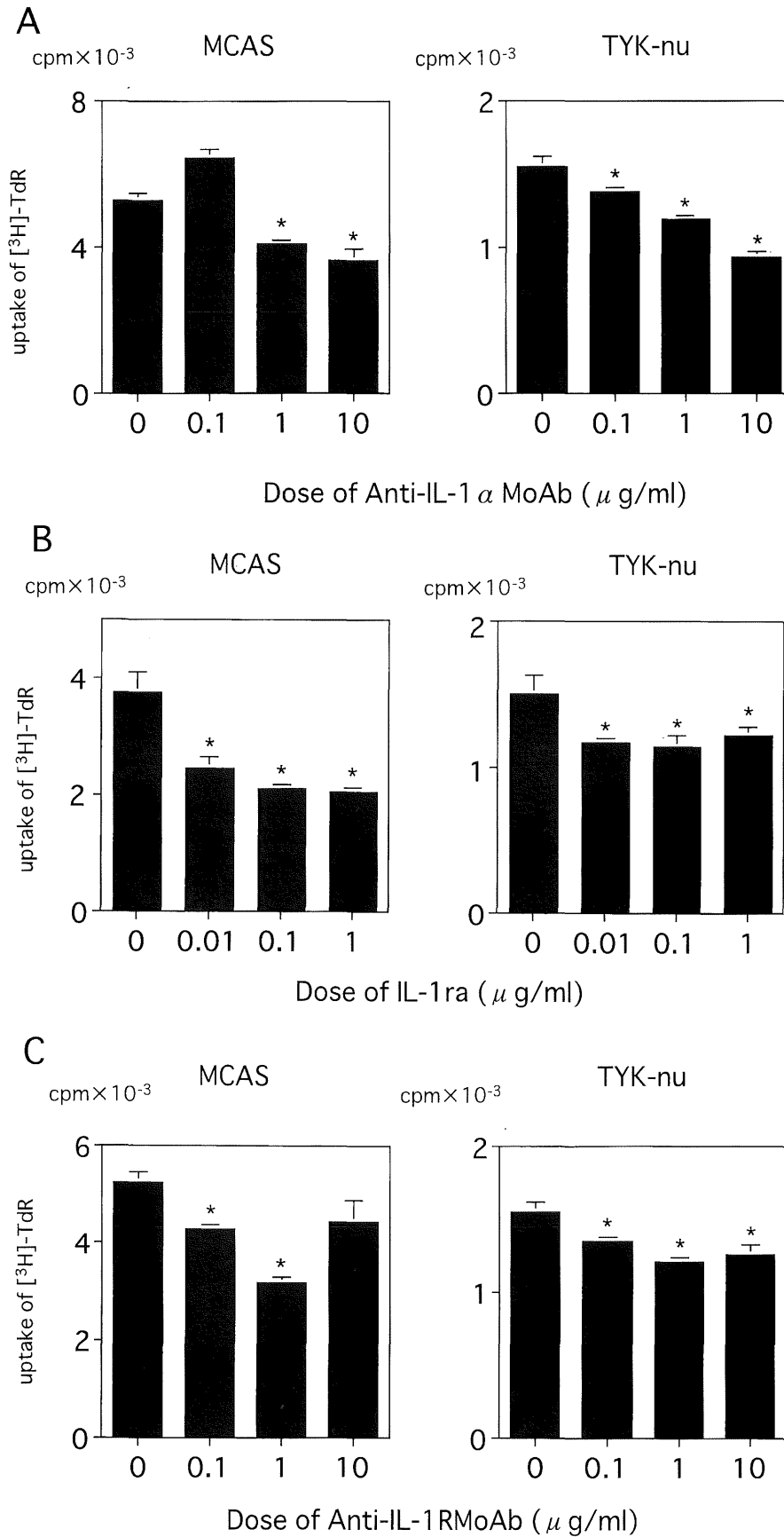
Fig. 5. Effect of anti-IL-1α MoAb (A), IL-1ra (B) and anti-IL-1RMoAb (C) on DNA synthesis of ovarian cancer cell lines, MCAS and TYK-nu. Cells (5×10^2 in MCAS and 1×10^3 in TYK-nu) were cultured for 72 hours with each agent at the concentrations indicated and labeled with [^{3}H]-TdR for the last 20 hours. Results are expressed as cpm of [^{3}H]-TdR uptake of tumor cells (mean ± SE of triplicate cultures); *significantly enhanced.

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by exogenous IL-1α was observed either by counting the viable cell number or by [^{3}H]-TdR incorporation (data not shown).

Growth inhibitory Activity of IL-1 Neutralizing Agents on Ovarian Cancer Cells

To assess whether IL-1α acts as an autocrine growth factor in ovarian cancer cells, neutralization studies were performed using anti-IL-1α MoAb, IL-1ra or anti-IL-1RMoAb. The spontaneous growth of MCAS and TYK-nu cells in [^{3}H]-TdR incorporation was inhibited by 23.9–30.4%, 11.1–40.7% with anti-IL-1α MoAb (Fig. 5A), by 34.6–44.2%, 19.2–23.1% with IL-1ra (Fig. 5B) and by 14.8–37.7%, 13.0–20.4% with anti-IL-1RMoAb (Fig. 5C), respectively.

Detection of IL-1 Receptor on Ovarian Cancer Cells

A Binding assay with ^{125}I-labeled IL-1α was

carried out to determine the presence of specific receptors for IL-1α on MCAS and TYK-nu cells. The specific binding of ^{125}I-IL-1α is displayed in Fig. 6A. The number of IL-1 receptor and their dissociation constants (*Kd*) were calculated by Scatchard plot analysis. The binding data revealed two linear regression lines, suggesting the presence of two classes of IL-1 receptors on each cell line. The *Kd* was 3.8×10^{-11}M and 2.5×10^{-10}M on MCAS and 4.9×10^{-11}M and 3.6×10^{-9}M on TYK-nu. The binding sites were 1.67×10^3 and 6.45×10^3 sites/cell on MCAS and 6.23×10^2 and 1.78×10^4 sites/cell on TYK-nu, respectively (Fig. 6B).

[^{125}I]-EGF Binding Studies

It has been reported that EGFR is commonly expressed and possibly plays a role in the autocrine growth mechanism in ovarian cancers[7]. We therefore studied the presence or absence of
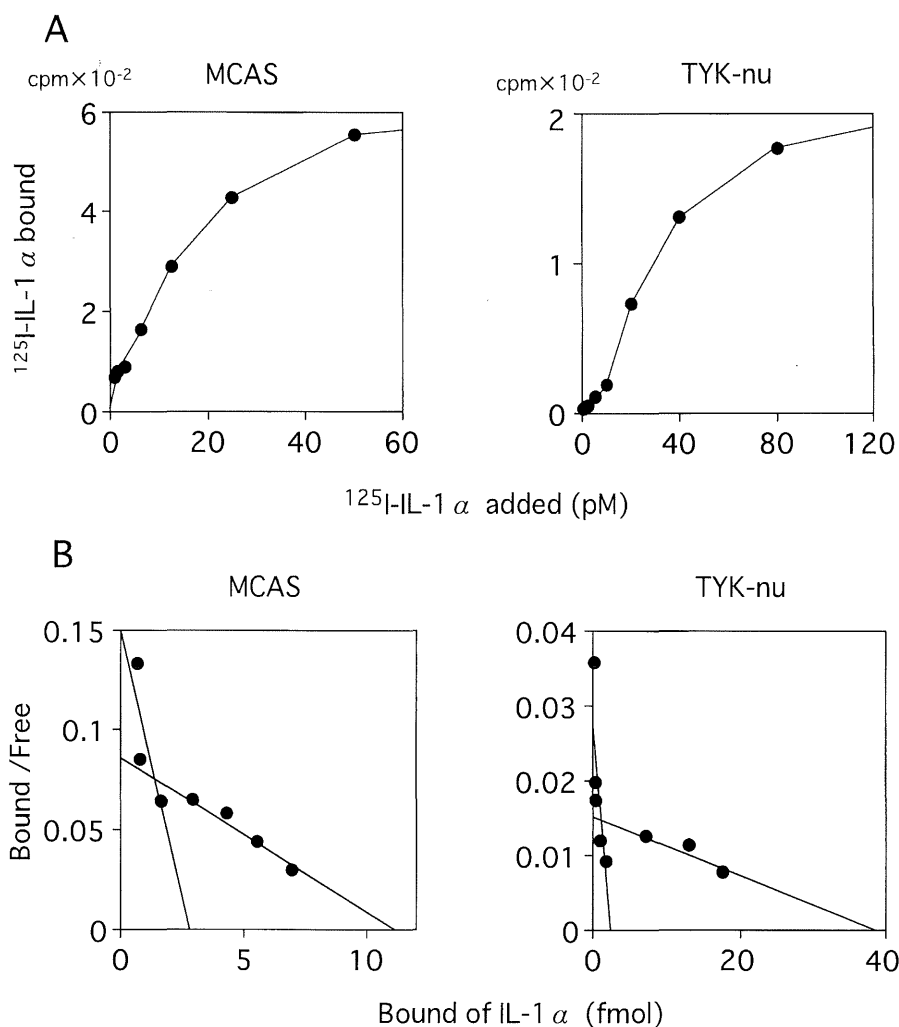

Fig. 6. Detection of IL-1R on ovarian cancer cell lines. A, specific binding of radiolabeled IL-1α to MCAS and TYK-nu. Specific binding is the difference between the binding of [^{125}I]-IL-1α in the presence or absence of 40 nM unlabeled IL-1α. Each point represents the mean of duplicate measurements. B, Scatchard plot analysis of [^{125}I]-IL-1α bound on ovarian cancer cells.

A

cpm×10⁻²

^{125}I-EGF bound (y-axis, values 0–4)

^{125}I-EGF added (nM) (x-axis, values 0, 200, 400)

B

Bound /Free (y-axis, values 0, 0.5, 1, 1.5)

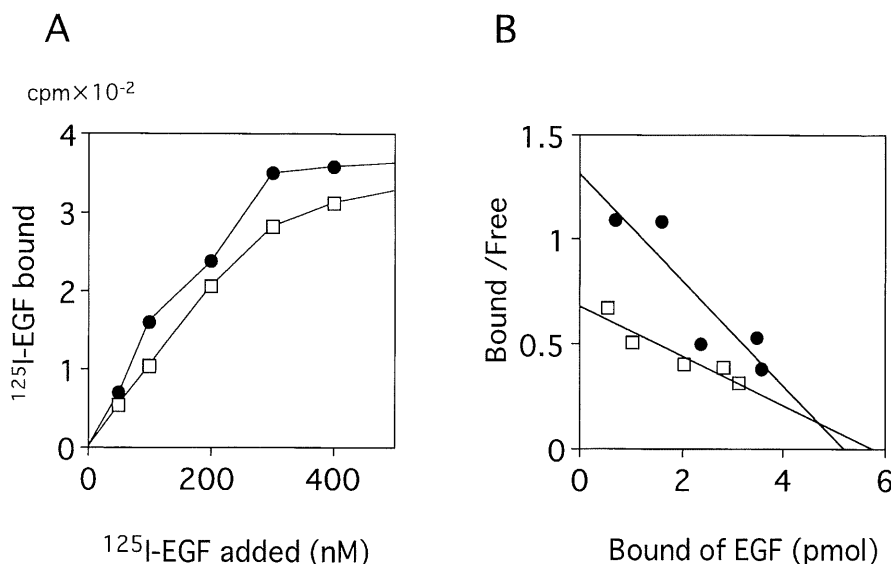Bound of EGF (pmol) (x-axis, values 0, 2, 4, 6)

Fig. 7. Detection of EGFR on MCAS cells. MCAS cells were cultured for 24 hours in the presence (●) or absence (□) of IL-1α (10 ng/ml) and the binding of [^{125}I]-EGF was performed by the same method described in Fig. 6. A, specific bindings of [^{125}I]-EGF to MCAS cells. B, Scatchard plot analysis of [^{125}I]-EGF bound on MCAS cells.

EGFR, and the relationship between the growth modulation of IL-1α and the expression of EGFR on MCAS and TYK-nu. [^{125}I]-EGF-specific bindings were observed and Scatchard plot analysis revealed single class of EGFR on both cells; Kd, 5.0×10^{-8}M on MCAS and 1.3×10^{-8}M on TYK-nu, binding sites, 3.23×10^{7} sites/cell on MCAS and 6.74×10^{6} sites/cell on TYK-nu. EGFR in these cells pretreated with 10 ng/ml IL-1α was compared with that of non-treated cells. Interestingly, the total number of binding sites did not change, but the increase of affinity was observed in MCAS; Kd, 1.7×10^{-8}M, pretreated with 10 ng/ml IL-1α (Fig. 7). EGFR in TYK-nu was not changed by the pretreatment of IL-1α in both the total number of binding sites and their affinity (data not shown).

DISCUSSION

There have been several reports that the abnormal growth of tumor cells is dependent on the various growth factors secreted by these cells themselves[25,27,28]. Li et al demonstrated that the inappropriate production of growth factors was a consequence of either genetic rearrangement, translocation or gene amplification, which finally resulted in the progression of ovarian cancer[12]. In the present study, eight ovarian cancer cell lines were examined for their growth regulation by IL-1α in an autocrine manner. Initially, we found that IL-1α mRNA was highly expressed in six out of eight ovarian cancer cell lines. In particular, in two lines, MCAS and TYK-nu, a high amount of IL-1α was secreted. IL-1β mRNA was also expressed in four out of the eight cell lines.

However, the secretion of IL-1β was not detected. The exogenous addition of IL-1α stimulated the growth of MCAS and TYK-nu cells. The maximum response was obtained at a concentration of 10 ng/ml of IL-1α. Furthermore, the spontaneous growth of these cells was inhibited by anti-IL-1α MoAb, IL-1ra and anti-IL-1RMoAb (Fig. 5A, B, C). In accordance with the effect of IL-1α on the growth of these cells, two classes of specific IL-1 receptors were expressed. Therefore, we concluded that the IL-1α produced by these cells stimulated the growth of these ovarian cancer cells, MCAS and TYK-nu, by an autocrine mechanism.

It is well known that IL-1 is a regulatory molecule for the induction of many types of immune and inflammatory responses. IL-1 was reported to be able to promote proliferation as a growth factor of several cell types such as T and B lymphocytes, immature myeloid precursors, epidermal cells, and fibroblasts, as well as several kinds of tumor cells such as acute myeloid leukemia cells, adult T-cell leukenia cells, thyroid carcinoma cells and gastric carcinoma cells[3,8,22,27,39]. In some ovarian cancer cells, it is reported that IL-1α or β is secreted and that their gene is constitutively expressed[12,34]. On the other hand, the cytocidal effect of IL-1 on several tumor cell lines including melanoma cells, uterine cervical carinoma cells, osteosarcoma cells and breast cancer cells has also been reported[4,11,21]. Patricia et al showed the antiproliferative effect of IL-1 on ovarian carcinoma cell lines and explained that its mechanism appeared to be mediated by the binding of IL-1 to type I IL-1 receptors on these cells[10]. Our

study confirms that IL-1α can also function as a growth factor for ovarian cancer cells by binding to type I IL-1 receptors, since its effect is blocked by IL-1ra which preferentially binds to type I IL-1 receptors and the anti-IL-1 receptor antibody used in this study recognizes type I IL-1 receptors. The mechanism by which IL-1α exerts a proliferative effect on these ovarian cancer cells is not known, but IL-1α may act to stimulate the production of other growth factors or the expression of their receptors. The expression of EGFR, which is a potent mitogen for a variety of cell types including ovarian cancer cells, on these cells was not changed in the total number of binding sites but their affinity was augmented by the addition of IL-1α in MCAS (Fig. 7)[14,15,33]. It is repoted that EGFR molecule consists of three domains and is regulated by intermolecular allosteric activation[36]. Tyrosine phosphorylation on EGFR may work in the mitogenic signal induced by the addition of IL-1α in MCAS.

Although the autocrine roles for IL-1α in the development of ovarian cancer cells *in vivo* are circumstantial at present, the work presented in this report substantiates that, at least at the *in vitro* level, IL-1α can play an autocrine role in the growth of ovarian cancer cells. Furthermore, it is possible that IL-1α plays an important role in the progression of this disease. The development of antagonists of this positive autocrine growth factor may provide a useful new approach to control the growth of cancer cells. Strategies to inhibit IL-1α expression and activity might provide a novel approach for the treatment of at least some ovarian cancer patients. It is necessary that the interactions of other cytokines, various growth factors and their receptors on these diseases are clarified in the future.

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