The Observation of Immunosuppressor(s) Derived from Cultured Tumor Cells and Its Partial Neutralization with OK-432

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Abstract

Malignant tumors such as brain tumors have been reported to be associated with immunosuppression caused by certain tumor-secreted cytokines. The reversion of tumor-derived immunosuppression has not been described. The use of OK-432, an immunomodulatory agent prepared from Su-strain of *Streptococcus pyogenes* A3, to activate peripheral blood mononuclear cells from a patient with glioblastoma multiforme has demonstrated a sharp rise in proliferative response. This proliferative response was compromised in the presence of living and irradiated autogeneic cancer cells. The conditioned media from cultured cells of glioblastoma multiforme, astrocytoma, and cholangiocarcinoma were tested for immunosuppressive ability. We found that conditioned media from 3 of 4 cases of glioblastoma, all 3 cases of astrocytoma, and 1 case of cholangiocarcinoma exhibited immunosuppressive activity toward the proliferative response of allogeneic peripheral blood mononuclear cells to phytohemagglutinin. This is the first report that cholangiocarcinoma produces soluble immunosuppressor(s). Our finding suggested that soluble substance(s) as well as direct cell-cell contact between tumor cells and mononuclear cells play roles in the observed tumor-derived immunosuppression.

Key word : Cancer, Immunosuppressor, Immunomodulator, Cholangiocarcinoma, Brain Tumor, Glioblastoma Multiforme, Astrocytoma

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Many published studies have reported the observation of suppressed immune responses in patients with advanced or relapsed cancer without the involvement of chemotherapy. This immune suppression involved cell-mediated immune response, the most important defense mechanism against the progression and dissemination of cancer[1]. The cytokine treatment at therapeutic level in plasma can be toxic to sensitive vascular riched organs and in some occasions can even promote tumor growth or metastasis[2,3]. All mentioned approaches have not addressed the issue of tumor-derived immunosuppressor(s). The existence of soluble immunosuppressors was reported in the condition media from diverse types of cultured cancer cells. These cultured cancer cells include choriocarcinoma [4], head and neck squamous cell carcinoma[5], guinea pig L2C leukemic cells[6], A549 human lung cancer cells[7,8], human melanoma cells[9], glioblastoma cells[10], and mouse plasmacytoma [11]. The suppressed immune function of tumor-bearing animals was reported to be recovered after tumor resection[12].

Cholangiocarcinoma is an attractive model for the study of secreted immunosuppressor(s) since the tumor is endemic in the northeastern part of Thailand where there is an association with liver fluke (*Opisthorchis viverrini*) infestation and nitrosamine ingestion. The human cell lines of cholangiocarcinoma were developed and characterized[13]. A hamster model for cholangiocarcinoma was also later developed [14]. We recently isolated cholangiocarcinoma from bile obtained through endoscopic examination. The study for the disturbance of host immunological functions by cholangiocarcinoma has not been established. We, therefore, examined the effect of OK-432, an immunomodulatory agent prepared from Su-strain of *Streptococcus pyogenes* A3, for the enhancement of T cell function against autogeneic tumor cells. OK-432 was developed for the treatment of various models of cancers[15,16]. As expected, OK-432 can activate the proliferation of peripheral blood mononuclear cells. However, the extent of proliferation in the presence of autogeneic glioblastoma cells was 34-67 per cent lower than those without tumor cells. This observation prompted us to investigate for the presence of immunosuppressor(s) derived from cultured cholangiocarcinoma and other solid tumors. Our preliminary results indicated the presence of soluble immunosuppressor(s) in the conditioned media from cholangiocarcinoma, advanced stage of astrocytoma including glioblastoma multiforme.

MATERIAL AND METHOD

Chemicals and Radiochemicals

Phytohemagglutinin (Sigma, St. Louis, USA) was stored as a solution of 100 μg/mL in serum-free RPMI-1640 medium at -20°C. [methyl-^{3}H]thymidine (5 Ci/mmol/5 mL, Amersham, Buckinghamshire, England) was prepared as a stock of 80 μCi/mL with serum-free RPMI-1640 medium and stored at -20°C. All tissue culture reagents were of tissue culture grade (Gibco BRL and HyClone). OK-432 (a gift from Chugai Pharmaceutical, Co. Ltd., Tokyo, Japan) was prepared as a stock solution of 5 KE/100 μL (5 mg/mL) in PBS and was freshly diluted with the complete RPMI-1640 medium to designated concentration prior to cellular treatment. MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) was prepared in RPMI-1640 as a 2 mg/mL solution.

Primary culture of tumor cells

Cultured cells of cholangiocarcinoma and all stages of astrocytoma including glioblastoma multiforme were prepared from surgical tissues collected from the neurosurgical operating rooms in Siriraj Hospital without the perturbation of regular treatment. Primary cultures were derived according to the techniques described by Westphal[17] with some modifications[18]. The obtained surgical specimens were minced with a surgical blade into small pieces, treated with 0.25 per cent trypsin-EDTA, and seeded at a density of 5,000-10,000 cells/cm^2 in 75-cm^2 tissue culture flasks. Cultured cells were propagated in growth medium (Dulbecco's modified Eagle medium (DMEM), 15 per cent fetal bovine serum, 1 mM sodium pyruvate, 1 mg/mL insulin, 0.55 mg/mL transferrin, 0.67 μg/mL sodium selenite, 0.1 mM non-essential amino acid solution, 2 mM *l*-glutamine, 50 unit/mL penicillin, 50 μg/mL streptomycin) at 37°C, 5 per cent CO_2. The cultured cells were morphologically homogeneous under the light

microscope after 4-week incubation. All samples of brain tumors were positively stained with glial fibrillary acidic protein (GFAP). Cholangiocarcinoma cells were prepared from metastatic lymph nodes of a subject pathologically confirmed for the diagnosis.

Isolation of peripheral blood mononuclear cells (PBMC)

Autogeneic peripheral blood mononuclear cells were prepared from the heparinized whole blood of a subject afflicted with glioblastoma multiforme, while allogeneic peripheral blood mononuclear cells were prepared from a consent healthy donor. About 10-12 mL of venous blood was drawn and contained in Vacutainer (Becton Dickinson) with sodium heparin. Plasma was isolated from whole blood through centrifugation at 1,400 × g (3,000 rpm) for 10 min. The remaining cell pellet was reconstituted with RPMI-1640 to 12 mL and layered on 2 mL IsoPrep® (Robbins Scientific Corp., Sunnyvale, CA). PBMC were isolated as a layer through gradient centrifugation at 1,400 × g (3,000 rpm) for 30 min at 18°C, and washed 3 times with 10-mL RPMI-1640 by centrifugation at 400 × g for 10 min at 18°C. The yield of PBMC was about 1×10^6 cells/mL of whole blood. The number of viable cells was at least 90 per cent as determined by trypan blue exclusion. Approximately 60 per cent to 70 per cent of PBMC were lymphocytes.

Proliferation Assay

The proliferative response of PBMC in the presence of phytohemagglutinin or activating stimuli involved the measurement of [^{3}H] thymidine incorporation into DNA that reflects DNA replication and hence cellular proliferation. This assay was used as a tool to verify the presence of tumor-derived immunosuppressor(s). PBMC's were dispensed into each well of a 96-well plate at 2×10^5 cells in 100 µL complete RPMI-1640. PHA (15 µg/mL) or OK-432 at indicated concentrations was added to constitute the final volume of 200 µL. The PBMC was incubated for 24-48 h in a humidified 37°C, 5 per cent CO_2 incubator. Cells were pulsed with 2 µCi [^{3}H]thymidine in the final 18 h before terminating the culture. Cells were harvested using an automated multiwell harvester that aspirated cells, lysed cells, and transfered DNA onto a filter paper, while allowing free [^{3}H]thymidine to pass through. Each row of the microtiter plate was filled and aspirated ten times to ensure complete cell transfer and complete removal of free thymidine. The filter was transferred to liquid scintillation vials, and counted for incorporated radiolabel with a liquid scintillation counter. The triplicate samples provided less than 20 per cent variation from the mean cpm.

Normalization of Cellular Metabolic Activity among Different Cell Lines

All cultured cell lines at graded cell numbers (1×10^3, 2×10^3, 4×10^3, 8×10^3, 1.6×10^4, and 3.2×10^4 cells) were processed for MTT assay in flat-bottom 96-well plate[19]. The obtained optical densities (Y-axis) were plotted against the cell numbers (X-axis). The observed linear portion of the plot was used to calculate the value of "*m*" (slope) and "*b*" (Y-intercept) of each cell line according to the expression of:

$$OD = mN + b$$

Where N = cell number of a particular cell line; and OD = optical density read from a well where cell numbers were designated as N. The value of "*b*" was similar in each cell line when tested in the same batch, but varied with different batches of MTT assay. This equation was used to locate the range of linear relationship between cell number and OD since the next experiment would employ cultured cells grown for 96 h on 96 well plate where the cell number could not be accurately counted. For determining cell number at the end of 96 h incubation (N_t), cultured cell lines (4×10^3 cells/well) were incubated with a complete RPMI-1460 medium on round-bottom 96-well plates at 37°C, 5 per cent CO_2 for 96 h. At the end of the incubation, each well was processed for MTT assay as described below. The OD read from the microtiter plate reader was used to calculate N_t according to the equation below. Assuming that the proliferation rate of each cultured cell line at this cell density was compatible with the first order kinetic equation, the

calculated value of N_t was further used to calculate the first order kinetic constant of cellular proliferation according to the following expression:

$$N_t = N_0 e^{kt}$$

The obtained calculated value of m, b, and k were used to calculate the area under curve (AUC) between OD and incubation time as the following expression:

$$AUC = \frac{mN_0}{k}(e^{kt} - 1) + bt$$

The calculated AUC between OD and time of each cultured cell line that was roughly proportional to accumulated cellular metabolism and, therefore, accumulated waste products excreted from cells to the medium. AUC obtained from each cell line was compared with the others. The AUC of one cell line with the minimum value would be used by other cell lines to back-calculate the appropriate starting cell number (N_0) to provide the equivalent AUC as the following expression:

$$N_0 = \frac{k(AUC - bt)}{m(e^{kt} - 1)}$$

Cultured cell lines with calculated N_0 were incubated with 200 μL complete RPMI-1640 at 37°C, 5 per cent CO_2 for 96 h. The conditioned media was then removed, filtered with 0.2 μm filter to remove cells and cellular debris prior to the test for their effects on allogeneic PBMC proliferation assay.

Collection of Conditioned media

The conditioned media from different types of cultured cells seeded at individually calculated starting cell number (N_0) were collected at the end of the 96-h incubation, centrifuged at 900 × g for 10 min to remove any remaining cells or cellular debris. The conditioned medium (50%) was added to each well of 96-well plate containing 2×10^5 fresh allogeneic human PBMC. The allogeneic PBMC in 50 per cent conditioned media were processed through the proliferation assay as described above.

MTT Colorimetric Assay to Measure Cellular Viability and Metabolic Activity

Cultured cells (100 μL) in 96-well plate were incubated with 2 μL of 2 mg/mL MTT/RPMI-1640 solution for 1 h in a humidified 37°C, 5 per cent CO_2 incubator[19]. The reaction was stopped by the addition of 100 μL of DMSO to each well. The microtiter plate was left at room temperature for completely dissolving all dark blue crystals. The plate was read for the absorbency of each well at 595 nm in a BioRad Model 3550 microtiter plate reader.

RESULTS

Immunomodulatory Effect of OK-432 on the Proliferation of Autologous Peripheral Blood Mononuclear Cells in the Presence of Primary Cultured Glioblastoma Multiforme Cells

We used proliferation assay as an indicator to determine the response of autogeneic PBMC's to OK-432, the immunomodulator, in the presence of tumor cells. The autogeneic PBMC's pre-treated with a gradient concentration of OK-432 for 48 h were confirmed for viability and immunologic competence by the proliferative response to nonspecific stimulation with PHA (Fig. 1). PHA at a range between 0.5-15 μg/mL had a favorable dose-response relationship to PBMC proliferation while at higher concentration would start to suppress the response (data not shown). The optimal concentration of PHA (15 μg/mL) was, therefore, employed throughout all presented experiments. The incubation of cultured tumor cells and the autogeneic PBMC's pretreated with OK-432 at a range between 0.001 – 0.01 mg/mL for 24 h (Fig. 1A) and 48 h (Fig. 1B) did not produce a different distribution pattern of proliferation. At a high concentration of OK-432 (0.1 mg/mL), the cellular proliferation was decreased after the pretreatment by 24 h and was virtually suppressed to undetectable levels by 48 h. OK-432 alone could stimulate the proliferation of autologous PBMC's in the absence of tumor cells. Furthermore, the proliferative response to PHA was increased by the addition of OK-432. It is noteworthy that the incubation of PBMC's with tumor cells, either as living cells or lethally irradiated cells, lessened the degree of OK-432 induced proliferation. The suppressive effect of living tumor cells at 4,000 cells/well (59 ± 7%) was more than that with 2,000 cells/well (45 ± 7%). Although irradiated tumor cells had immunosuppressive effect, the degree of suppression was less than that of living tumor cells.

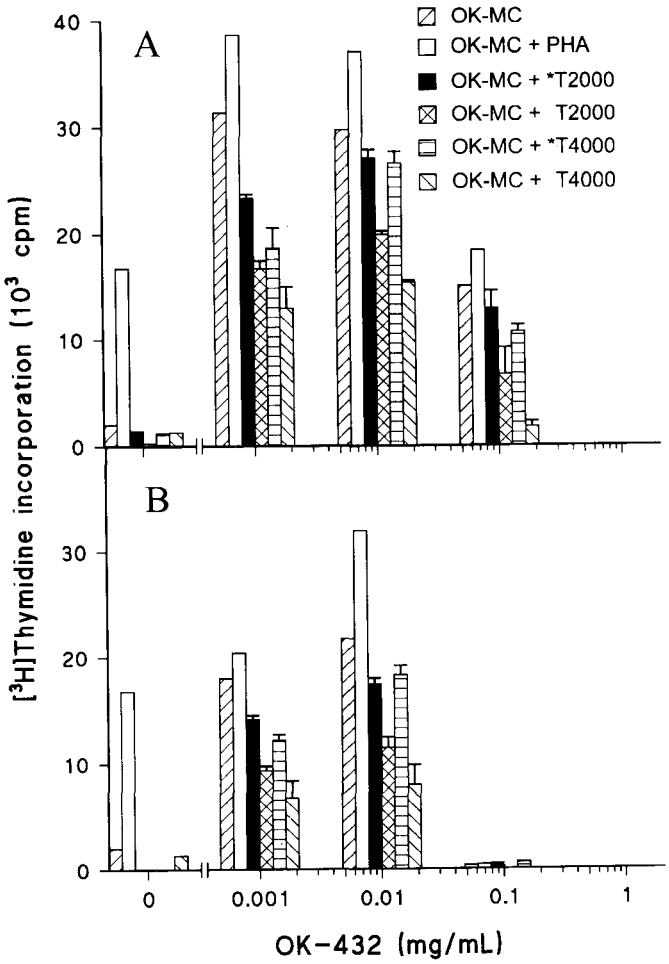

Fig. 1. The proliferation of peripheral blood mononuclear cells (PBMC's, 2×10^5 cells/200 μL/well) pre-incubated with a series of gradient concentrations of OK-432 (0, 0.001, 0.01, 0.1 mg/mL) for 48 h in the presence of either living or irradiated autogeneic cultured cells of glioblastoma multiforme. The OK-432-pre-incubated PBMC's (OK-MC's) were incubated with 2,000 or 4,000 cells of either living tumor cells (T) or irradiated tumor cells (*T) for 24 h (panel A) or 48 h (panel B). Cells were pulsed with 2 μCi [^{3}H]thymidine in the final 18 h before terminating the culture. The integrity of cellular function was affirmed by the demonstration of proliferative response of OK-MC to phytohemagglutinin (PHA, 15 μg/mL). OK-MC's alone without autogeneic tumor cells or PHA served as a baseline control. Each bar represents the mean value of triplicate samples.

Normalization of Metabolic Activity between Different Cultured Cells

The observation of immunosuppressive effect of tumor cells led us to investigate the possibility that this effect could at least be mediated through cellular excretory products. However, the immunosuppressive effect of specific immunosuppressor(s) needed to be differentiated from the nonspecific effect of general metabolic products. We assumed that the excretory waste products were accumulated in medium in proportional to accumulated cellular metabolic

activity and hence the accumulated MTT metabolic activity. We, therefore, conducted a set of experiments in which different cultured cells were examined for MTT metabolic assay. Active mitochondria in viable cells will cleave the pale yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to form a dark blue formazan product. The color can be readily detected with a microtiter plate reader[19]. We observed a linear relationship between the viable cell count and the MTT absorbance at a range of optical absorbency between 0.03-0.30, which corresponded to a range of approximately between 0 to 2×10^4 cultured cells/well on a 96-well plate. By working on the number of different cultured cells starting at 1×10^3, 2×10^3, and 4×10^3 cells/well at time 0 and propagated exponentially until 96 h, the MTT assay was performed on these cells at 0 h and at 96 h. The accumulated MTT activity can be represented by the calculated area under the curve (*AUC*) between OD and incubation time with the assumption that cellular proliferation followed first order kinetic. An appropriate *AUC* was chosen to back-calculate N_0 that would give rise to cell numbers that would still be in the linear range of optical absorbency in MTT assay after 96 h (Table 1). Cultured fibroblast starting at 3,650 cells/well as well as other cell types starting at individual N_0 calculated from equivalent *AUC* stayed healthy on 96-well plate at the end of 96-h incubation as determined by trypan blue exclusion method (data not shown). At higher N_0, viability of some cell types was compromised.

Table 1. The calculated values of doubling time and selected normalized N_0 of different cultured tumor cells.

Sample	Doubling time (day)	Normalized N_0 (cells/well)
Cholangiocarcinoma (C3)	3.99	1,725
Astrocytoma (G44)	1.64	2,224
Astrocytoma Grade I (G17)	2.57	2,160
Astrocytoma Grade II (G34)	7.55	1,166
Glioblastoma multiforme (G19)	1.41	673
Glioblastoma multiforme (G41)	3.43	5,395
Glioblastoma multiforme (G47)	4.71	1,935
Fibroblast (F1)	1.43	3,045
Fibroblast (F2)	1.62	3,654

Immunosuppressive Effect of Conditioned Media from Various Cultured Tumor Cells

We observed suppression of response to PHA in wells containing conditioned media from 2 samples of glioblastoma multiforme, 3 samples of astrocytoma, and a sample of cholangiocarcinoma (Fig. 2). The degree of suppression was between 97-99 per cent, almost totally abrogate the response to PHA. The suppression of PBMC also could be readily observed in wells without PHA, but with less significance. However, conditioned media from a sample of glioblastoma multiforme and both samples of primary cultured fibroblast did not exhibit any observable suppressive effect.

DISCUSSION

The cell proliferation assay is a very useful method for monitoring the immunological status *in vitro*. The process of proliferation in response to external stimuli is a very complex process that is not well understood. This process involves the delivery of signals from the cell membrane, activation of intracellular endogenous mediators, transcription of multiple genes, DNA and protein synthesis, and cell division. PHA, the most widely used mitogen, was found to mediate its action *via* its high affinity binding with α/β Chains of T cell receptor on T cells [20]. The action site of OK-432 on PBMC is not yet known but the interaction caused the PBMC to release several cytokines such as interleukin (IL)-1β, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, and TNF-α[21,22]. The additive proliferation in response to PHA and OK-432 indicated that these two reagents exerted at least some different immunostimulatory pathways. The proliferative response to PHA was extremely sensitive to suppression (97-99%) with conditioned media from cultured cancer cells. The proliferative response of PBMC to OK-432, although sensitive to the conditioned media, was suppressed to a much lesser degree. The maximal observed suppression to OK-432-activated PBMC was about 50-60 per cent in the presence of living

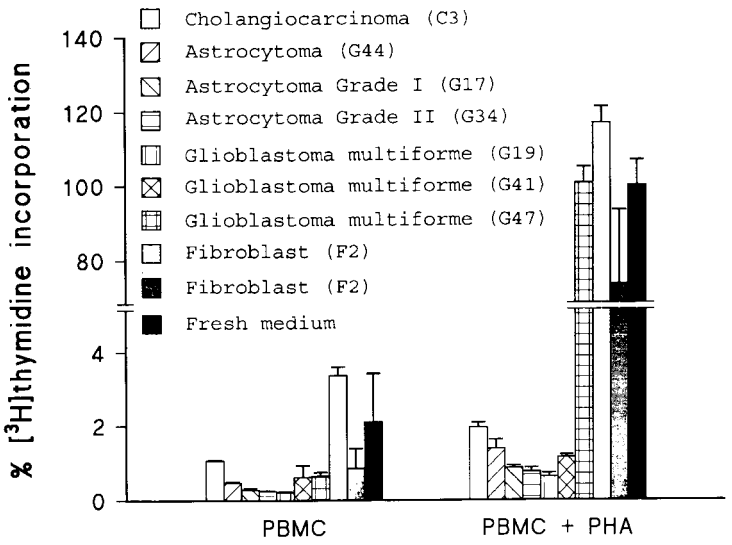

Fig. 2. The suppression of the proliferative response of allogeneic PBMC's to PHA by the addition of conditioned media from tumor cell cultures, but not from human fibroblast primary culture. Tumor cells and human fibroblasts were grown for 96 h with the appropriate starting cell counts calculated based on the normalization of accumulated MTT conversion activity. The fresh allogeneic PBMC (2×10^5 cells/well) from a healthy donor were prepared and incubated for 72 h with 15 μg/mL PHA in 200 μL of 50 per cent conditioned media collected from each type of cultured tumor cells or fibroblasts. Control wells contained fresh PBMC's and PHA in fresh complete RPMI-1640. Those wells containing fresh PBMC without PHA provided baseline proliferative responses to the conditioned media. The experiments were performed as 2 sets on separate occasions and plotted on the same scale based on cpm obtained from samples in fresh media of individual set. The first set included samples in conditioned media of G41, G44, C3, and F1 with 277,623 cpm obtained from samples in fresh media. The second set included samples in conditioned media of G17, G19, G34, G47, and F2 with 452,451 cpm obtained from samples in fresh media. Each bar represents the mean value of at least three samples.

cancer cells, and about only 20-30 per cent in the presence of irradiated cancer cells. These demonstrated the potential beneficial action of OK-432 in counteracting immunosuppression.

The observation that the irradiated cells incubated with autogeneic PBMC in fresh medium (Fig. 1, bar 3 and bar 5 at 0.001 and 0.01 mg/mL OK-432) also suppressed the proliferation of the latter, served as evidence that (1) tumor cells could utilize a direct cell-cell contact as a mean to mediate the suppression, or (2) some toxic waste products could be released from apoptotic tumor cells. The immunosuppression based on direct cell-cell interaction should involve the signal attached on the surface of the irradiated cancer cells in which Fas ligand is a possible candidate. The nature of immunosuppressor(s) derived from tumor cells either as a soluble form in the conditioned medium or a membranous form attached to the surface of tumor cells are under investigation.

The possibility that this suppression could be generated by nonspecific metabolic waste products of any cultured cells could not be excluded. We then normalized every type of cultured cells for equivalent *AUC* generated

from data of MTT assay and calculated individual appropriate starting cell number. MTT assay has generally been used as an alternative method to monitor viable cell activity[19]. The use of accumulated MTT activity to represent the amount of secreted waste products was devised by us under the assumption that the metabolic waste products were produced in proportion to accumulated cellular metabolic activity, hence accumulated MTT activity. The normalization for accumulated MTT activity in individual cell type has made the direct comparison of immunosuppressive effects produced from different cell types possible. The conditioned media collected from these cell lines at the end of 96 h were, therefore, assumed to contain equivalent amount of excretory waste products. Our observation that the conditioned medium from cholangiocarcinoma could suppress PBMC proliferation is the first report that cholangiocarcinoma has an immunosuppressive effect. The observation that conditioned medium from fibroblast (F1) had an additional immunostimulatory effect (16%) over the fresh medium suggested that some immunostimulatory cytokines could be released from fibroblast. However, subsequent experiments revealed comparable effect of conditioned medium from fibroblast (F1) to fresh medium (data not shown). The possibility that xenogeneic antigens contained in fetal bovine serum could confound human PBMC proliferation in this set of experiments has been excluded since all samples of conditioned and fresh media contained an equal amount of fetal bovine serum. The notion that nutrient deprivation in 50 per cent conditioned media could compromise the PBMC proliferation has been excluded. The 50 per cent growth medium in glucose-deprived Hanks' balanced salt solution did not compromise the PBMC proliferation (118.4 ± 1.1%) from the control with 100 per cent fresh growth medium (100 ± 3.1%) under the same described condition.

Several secreted biological products have been reported as immunosuppressors. They consist of transforming growth factor β (TGF-β), p15E protein, IL-8, IL-10, insulin-like growth factor 1 (IGF-1), prostaglandin E_2 (PGE_2), mucins, suppressive E- receptor (SER), immunosuppressive acidic protein (IAP), and adhesion molecules [1]. Among these secreted products, TGF-β is the most potent immunosuppressor. TGF-β can be isolated from several types of cultured tumor cells as well as in the plasma of the cancer patients [23-25]. Antagonizing TGF-β may have a therapeutic potential in reversing immunosuppression[26,27]. PGE_2 produced from tumor tissue or induced macrophage were reported to promote the process of tumorigenesis[10,28]. Therefore, nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin may play a role in the improvement of immune function [29]. IL-8, an angiogenic cytokine, can promote tumor growth[3]. IL-10 was reported to be a mediator of immunosuppression in squamous cell carcinoma of the head and neck[30], fibrosarcoma[28] and human bronchogenic carcinoma [31].

The extent of immunosuppression may be lessened by the addition of certain cytokines. TNF-α and GM-CSF were reported to reverse the T cell suppression of pulmonary alveolar macrophage[32,33]. Vitamin D or a combination of IFN-γ, TNF-α and IL-2 improved the immune function in mice bearing lung carcinoma[34-36]. Several protocols of immunotherapy as well as gene therapy aim at the restoration of host immune responses. These protocols consist of up-regulating tumor-associated antigen, HLA presentation, and injecting immunostimulatory cytokines (e.g., IFN-γ, tumor necrosis factor α (TNF-α), IL-1, IL-2, etc.). However, these approaches have limited success[1]. The response of autogeneic PBMC to OK-432 demonstrated the possibility for OK-432 to reverse the immunosuppression which occurred in cancer patients. The immunosuppressive effect of cancer cells to autogeneic PBMC's was clearly reversible since the activation by OK-432 could at least partially counteract the tumor-secreted immunosuppressors. The clinical use of OK-432 may be advantageous to other models of immunotherapy that require intact immune response for effective outcomes.

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การตรวจพบความสามารถกดภูมิคุ้มกันโดยเซลล์มะเร็งเพาะเลี้ยงและการต่อต้านการกดภูมิคุ้มกันด้วยโอเค–432

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มีรายงานการตรวจพบภาวะภูมิคุ้มกันบกพร่องในโรคมะเร็งสมองซึ่งพบว่าเกิดโดยฤทธิ์ของสารคัดหลั่งจากเนื้อเยื่อมะเร็งเอง แต่ยังไม่มีรายงานถึงการต่อต้านภาวะภูมิคุ้มกันบกพร่องจากมะเร็ง การใช้โอเค–432 ซึ่งเป็นยาเสริมภูมิคุ้มกันที่เตรียมจาก Su–Strain ของเชื้อ *Streptococcus pyogenes* A3 เพื่อกระตุ้นเม็ดเลือดขาวที่เตรียมจากของผู้ป่วยมะเร็งสมองชนิด glioblastoma multiforme ได้ผลทำให้เม็ดเลือดขาวชนิดนิวเคลียสกลีบเดียวที่เตรียมจากหลอดเลือดส่วนปลายมีการแบ่งตัวสูงขึ้น แต่ถ้ามีเซลล์มะเร็งเพาะเลี้ยงทั้งที่ผ่านและไม่ผ่านการฉายรังสีร่วมในภาชนะเดียวกันแล้วจะลดอัตราการแบ่งตัวนี้ การศึกษาต่อในน้ำยาเลี้ยงเซลล์จาก 3 ใน 4 รายของ glioblastoma multiforme จากทั้ง 3 รายของ astro–cytoma และจาก cholangiocarcinoma 1 ราย พบว่ามีคุณสมบัติกดภูมิคุ้มกันโดยลดการแบ่งตัวของเม็ดเลือดขาวลงอย่างมาก ผลงานวิจัยนี้ได้บอกถึงการที่เนื้อเยื่อมะเร็งสามารถหลั่งสารกดภูมิคุ้มกันรวมทั้งยังอาจใช้การสัมผัสระหว่างผิวเซลล์เพื่อยับยั้งการแบ่งตัวของเม็ดเลือดขาวได้

คำสำคัญ : มะเร็ง, สารกดภูมิคุ้มกันจากเนื้อเยื่อมะเร็ง, มะเร็งเยื่อบุทางเดินน้ำดี, มะเร็งสมอง

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