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Enhanced apoptosis of squamous cell carcinoma cells by interleukin-2-activated cytotoxic lymphocytes combined with radiation and anticancer drugs

T. Yamamoto *, K. Yoneda, E. Ueta, S. Doi, T. Osaki

Department of Oral Surgery, Kochi Medical School Kohasu, Oko-cho, Nankoku-city, Kochi 783-8505, Japan

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Abstract

Induction of potent apoptosis is required in cancer therapy. We examined the combination effect of interleukin-2-activated lymphocytes (LAK cells) and anticancer drugs or gamma (γ)-rays on the induction of apoptosis in an established oral squamous cell carcinoma cell line (OSC-3 cells). By pretreatment of OSC-3 cells with ^{137}Cs (5 Gy), 5-fluorouracil (5-FU) (0.5 μg/ml) or *cis*-dichlorodiammine-platinum (CDDP) (5 μg/ml), the activation of bid and caspase-3 by LAK cells was strongly increased and associated with an enhanced degradation of poly-(ADP-ribose) polymerase (PARP) and/or nuclear mitotic apparatus protein (NuMA) and the increased fragmentation of DNA. The LAK cell–enhanced caspase-3 activity in the pretreated OSC-3 cells was decreased to approximately 70% and 40% of the control by the addition of Z-AAD-CMK (a granzyme B inhibitor) and neutralising monoclonal antibody to Fas antigen (αFas-IgG), respectively. The combined treatment-induced DNA fragmentation was suppressed by approximately 20% and 30% of the control by the addition of Z-AAD-CMK and αFas-IgG, respectively, in the co-culture system. While Ac-DEVD-CHO (a caspase-3 inhibitor) suppressed the DNA fragmentation levels to approximately half and this was similar to the amount of suppression that was obtained by the addition of both αFas-IgG and Z-AAD-CMK. In addition, LAK cell-activated bid may have increased the intracellular reactive oxygen intermediates (ROI) level and induced a decrease of mitochondrial membrane potential. These influences by LAK cells were enhanced when OSC-3 cells were pretreated with each anticancer drug or ^{137}Cs. Furthermore, the increase of ROI by LAK cells was suppressed by αFas-IgG and Z-AAD-CMK to approximately half the level of the control. These results indicate that anticancer drugs and γ-rays prime squamous cell carcinoma cells to be susceptible to apoptosis by LAK cells, that LAK cell-induced apoptosis largely depends on the activation of caspase-3 by the Fas/Fas-ligand signal and granzyme B, and that LAK cells induce ROI in the target cells, which is largely mediated by Fas and granzyme B.

Keywords: Apoptosis; Caspase; Granzyme B; Fas; LAK cells; Reactive oxygen intermediates; Squamous cell carcinoma cells

1. Introduction

Induction of tumour cell differentiation and apoptosis have gradually become targets in cancer therapy [1,2]. The all-*trans*-retinoic acid has been shown to have a substantial ability to induce differentiation in acute promyelocytic leukaemias, and the therapeutic effects of other agents capable of inducing differentiation such as the interferons, 13-*cis*-retinoic acid, 22-oxa-1α, 25-dihydroxyvitamin D_3 and vesnarinone have also been reported in solid tumours including breast cancers, colon cancers, neuroblastomas and salivary gland tumours [3–7]. Therapies capable of inducing differentiation and apoptosis lead to the preservation of the function of the organs, and such function-preserving therapy is recommended in oral carcinomas [8]. However, little data concerning such therapy have been reported in squamous cell carcinomas [9,10].

Differentiation and apoptosis are induced not only by the above differentiation-inducing agents, but also by chemotherapeutic drugs and gamma (γ)-rays [9–14]. In addition, cytotoxic lymphocytes such as major histocompatibility complex class I-restricted cytotoxic T lymphocytes, tumour-infiltrating lymphocytes and lymphokine-activated killer (LAK) cells can induce tumour cell differentiation and apoptosis, although the signal

* Corresponding author. Tel.: +8-888-80-2423; fax: +8-888-80-2424.

E-mail address: yamamott@kochi-ms.ac.jp (T. Yamamoto).

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pathways involved differ somewhat from each other [15–19]. As is well known, induction of differentiation requires a G_1-arrest of the cells, but apoptosis can be induced in all cell cycle phases [20–22]. Therefore, therapy that induces apoptosis may be more effective in the treatment of malignant tumours than drugs that induce differentiation.

Apoptosis is characterised by marked morphological changes such as membrane blebbing, chromatin condensation, nuclear breakdown and internucleosomal DNA fragmentation [2]. Caspases, cysteinyl aspartate-specific proteases, play an important role in signalling the induction of apoptosis [23–25]. The caspases are activated through two signal transduction pathways [26–28]. One of the pathways starts from the Fas-associated death domain (FADD) which is activated by the binding of Fas with Fas-ligand (Fas-L). This leads to the assembly of a signal transduction complex that results in activation of the caspase cascade [29]. In this cascade, FADD-associated caspase-8 is activated first resulting in cleavage of the downstream caspases and finally, in activation of caspase-3. The other pathway starts in the mitochondria [30,31]. Anticancer agents such as bleomycin, *cis*-dichlorodiammineplatinum (CDDP), 5-fluorouracil (5-FU) and γ-rays induce reactive oxygen intermediates (ROI) in the internal mitochondrial membrane and these ROI decrease the potential of the external mitochondrial membrane, which leads to release of cytochrome c [32–35].

Chemotherapeutic drugs and γ-rays appear to induce tumour cell apoptosis via the release of cytochrome c, while cytotoxic lymphocytes induce apoptosis by the Fas/Fas-L system and perforin/granzyme B. Therefore, strong apoptotic signals are expected when tumour cells are treated with cytotoxic lymphocytes and the apoptosis-inducing anticancer drugs or γ-rays. Frost and colleagues have reported that prostate carcinoma cell lines are sensitised for lymphocyte-mediated apoptosis via the Fas/Fas-L pathway by their pretreatment with anticancer drugs [17]. However, co-operation of anticancer agents and γ-rays with lymphocytes has not been investigated in squamous cell carcinoma cells. In the present study, we investigated whether LAK cell-induced apoptosis of squamous cell carcinoma cells is augmented by pretreatment with chemotherapeutic drugs such as 5-FU, CDDP and γ-rays.

2. Materials and methods

2.1. Cell line

An OSC-3 cell line was established from an oral squamous cell carcinoma and cultured in Dulbecco's modified Eagle's essential medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics. Daudi cells, derived from Burkitt's lymphoma, were grown in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium (Nissui Pharmaceutical Co., Ltd) supplemented with 10% (v/v) heat inactivated FBS, 2 mM L-glutamine and antibiotics. OSC-3 cells can be transplanted into nu/nu mice and the expressed *TP53* gene is mutated at codon 176, Cys to Phe.

2.2. Reagents

The cytotoxic anti-Fas monoclonal antibody (MAb) (αFas-IgM, clone CH-11) and neutralising MAb of Fas (αFas-IgG, clone ZB4) were purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Caspase-3 inhibitor, Ac-DEVD-CHO and granzyme B inhibitor, Z-AAD-CMK were obtained from Calbiochem (Cambridge, MA, USA).

2.3. LAK cell induction

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised peripheral blood of healthy donors by the Ficoll-Paque (Pharmacia Fine Chemicals, NJ, USA) gradient method. The obtained PBMC were washed twice with phosphate-buffered saline (PBS), resuspended in RPMI1640 medium containing interleukin-2 (IL-2, 75 U/ml, Shionogi Pharmaceutical Co. Osaka, Japan) and 10% (v/v) heat-inactivated human AB serum, and cultured for at least 7 days at 37°C in plastic flasks. The mean cytotoxic activities of the induced LAK cells against Daudi cells were 50–75% at an effector cell:target cell (E:T) ratio of 20:1.

2.4. Cell growth inhibition assay

OSC-3 cells were exposed to the indicated doses of γ-rays using a ^{137}Cs source and cultured in medium for 48 h at 37°C or were cultured in medium containing the indicated doses of 5-FU (Sigma-Aldrich Chemicals, Tokyo, Japan) or CDDP (Sigma) for 48 h at 37°C. The cells were then washed and cultured with or without LAK cells at an E:T ratio of 10:1 for 48 h. After the cultivation, viable OSC-3 cells were counted by excluding non-viable cells which were positively stained with trypan blue. In the trypan blue exclusion, squamous OSC-3 cells were morphologically distinguished from LAK cells which were round and small. Per cent inhibition of cell proliferation was calculated using the following formula: % of growth inhibition = $[1-(Nt-No)/(Ns-No)]\times 100$, where No is the number of seeded OSC-3 cells, Ns is the number of OSC-3 cells cultured without LAK cells for 48 h, and Nt is the number of OSC-3 cells cultured with or without LAK cells for 48 h after each pretreatment which is shown in the figure legends.

2.5. DNA fragmentation assay

The size distribution of target cell DNA was investigated. Briefly, OSC-3 cells were labelled with 15 μCi/ml [^{125}I]IdUrd (Dupont NEN, Boston, MA, USA) for 1.5 h at 37°C and washed with warm medium. The labelled OSC-3 cells were exposed to 10 Gy of γ-rays using a ^{137}Cs source and cultured in medium for 48 h at 37°C or were cultured in medium containing 5-FU (0.5 μg/ml) or CDDP (5 μg/ml) for 48 h at 37°C. The cells were then washed and cultured with or without LAK cells at an E:T ratio of 10:1 for 18 h. After cultivation, the cells were harvested by centrifugation at 200 *g* for 10 min and resuspended in 2.5 ml of buffer (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.6, 0.2% (v/v) Triton X-100, 0.2 mM dithiothreitol (DTT)). The cells were homogenised and layered over 2 ml of 25% (v/v) glycerol containing 10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$ and 0.2 mM DTT. The tubes were centrifuged at 200 *g* for 10 min and the nuclear pellets were lysed by the addition of 2 ml of buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5% (v/v) Nonidet (N)P-40, and 0.5% (w/v) sodium dodecyl sulphate (SDS)). DNA from lysed nuclei and culture supernatants was extracted twice with phenol/chloroform. After the extraction, DNA was subjected to agarose gel electrophoresis. [^{125}I]IdUrd-labelled target cell DNA was visualised by autoradiography.

In addition to the analysis of target cell DNA size, per cent DNA fragmentation was assayed by measuring [^{3}H]TdR release. Briefly, OSC-3 cells were labelled with 25 μCi/ml [^{3}H]TdR (DuPont NEN) for 24 h at 37°C, washed with warm medium, and incubated in fresh medium for 4 h at 37°C. The labelled OSC-3 cells were pretreated as described above and cultured with or without LAK cells at an E:T ratio of 10:1 for the indicated times. After cultivation, the supernatants were collected, centrifuged at 12 000 *g* for 10 s, and retained for later radioactivity counting (Fraction S). The cells were treated with ice-cold lysis buffer (25 mM sodium acetate, pH 6.6) for 1 h at 4°C. DNA from these cells was separated by centrifugation at 14 000 *g* for 15 min into fragmented low molecular weight (m.w.) (Fraction L) and intact high m.w. (Fraction H) fractions. The radioactivities of these fractions were determined using a liquid scintillation counter. The DNA fragmentation was calculated as a percentage of specific DNA fragmentation, as determined by the following formula: {[experimental counts per minute (cpm) (S + L)/experimental cpm (S + L + H)]-[spontaneous cpm (S + L)/spontaneous cpm (S + L + H)]} × 100. Spontaneous release was determined in non-treated cells.

2.6. Phosphatidylserine externalisation assay

After pretreatment with each anticancer agent for 48 h as described for the cell growth inhibition assay, OSC-3 cells were co-cultured with LAK cells for 6 h at an E:T ratio of 5:1. The cultured cells were stained with an annexin-fluorescein isothiocyanate (FITC) apoptosis detection kit (Calbiochem) and subjected to flow cytometric analysis on a Becton Dickinson FacScan using ImageQuant software (Becton Dickinson, San Jose, CA, USA).

2.7. Western blotting

After each treatment, cells were washed in ice-cold PBS and lysed for 10 min in 50 mM Tris–HCl, pH 7.6, containing 1% (v/v) NP-40, 300 mM NaCl, and protease inhibitors (3 μg/ml leupeptin, 3 μg/ml aprotinin and 2 mM phenylmethylsulphonyl fluoride (PMSF)). Proteins from these cells were loaded in each lane and electrophoretically separated on a 10% polyacrylamide gel in the presence of SDS and reducing conditions. Following SDS–polyacrylamide gel electrophoresis (PAGE), proteins were transferred to PVDF membrane (Immobilon, Millipore Corporation, MA, USA) by semi-dry electroblotting. The loading and transfer of equal amounts of protein was confirmed by staining the PVDF membrane with amido black. The membrane was blocked overnight with 5% (w/v) non-fat dry milk powder in PBS and then incubated for 1 h with anti-caspase-3 (CPP32) MAb (Calbiochem), anti-poly-(ADP-ribose) polymerase (PARP, Biomol Research Laboratories, Inc., PA, USA), anti-nuclear mitotic apparatus protein (NuMA, Calbiochem) MAb and anti-bid goat polyclonal Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was washed three times with PBS containing 0.05% (v/v) Tween 20 and incubated with peroxidase-conjugated, affinity-purified rabbit anti-mouse or anti-goat IgG for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining (ECL) following the manufacturer's instructions (Amersham Life Science, Tokyo, Japan).

2.8. Measurement of caspase-3 protease activities

Caspase-3 protease activities were assayed by the cleavage of Ac-DEVD-AMC (Biomol Research Laboratories). Briefly, the treated cells were sedimented at 200 *g* for 10 min, washed twice with PBS, and resuspended in buffer C (25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). After a 20 min incubation on ice, the cells were lysed with 20–30 strokes in a tight-fitting Dounce homogeniser. Following removal of nuclei by sedimentation at 800*g* for 10 min, 0.5 mM EDTA was added to the supernatant and a further sedimentation step was carried out at 280 000 *g* for 60 min in a Beckman TL-100 ultracentrifuge. After addition of DTT to a final concentration of 2 mM, the supernatant (cytosol) was frozen in 50 μl aliquots at

−70°C. Aliquots containing 50 μg of cytosolic protein in 50 μl of buffer C were diluted with 225 μl of freshly prepared buffer D (25 mM Hepes, pH 7.5, 0.1% (w/v) CHAPS, 10 mM DTT, 8 μg/ml aprotinin and 1 mM PMSF) containing 100 μM substrate and incubated for 2 h at 37°C. Reactions were terminated by the addition of 1.225 ml of ice-cold buffer D. Fluorescence was measured in a fluorometer using an excitation wavelength of 360 nm and emission wavelength of 475 nm. Reagent blanks containing 50 μl of buffer C and 225 μl of buffer D were incubated at 37°C for 2 h and then diluted with 1.225 ml of ice-cold buffer D.

2.9. Intracellular ROI levels

The production of ROI was estimated fluorometrically using an oxidation-sensitive fluorescent probe, hydroethidine (HE, Molecular Probes, Eugene, OR, USA). Briefly, the treated cells were washed three times in 5 mM Hepes-buffered saline, pH 7.4, incubated in Hepes-buffered saline containing 0.1 μM HE for 15 min, and analysed on a FACS flow cytometer.

2.10. Mitochondrial membrane potential and mitochondrial size assays

Cells were incubated in the culture medium containing Rh123 (2 μM, Molecular Probes) for 30 min at 37°C. The cells were then washed with PBS and resuspended in PBS. The mitochondrial membrane potential (ψ_m) was analysed on a FACS flow cytometer. Mitochondrial fractions were prepared by resuspending cells in 0.8 ml ice-cold buffer A (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 2 mM PMSF, 8 μg/ml aprotinin, 2 μg/ml leupeptin (pH 7.4)). The cells were passed through a tight-fitting Dounce homogeniser. Unlysed cells and nuclei were pelleted by spinning for 10 min at 750*g*. The supernatants were centrifuged at 10 000*g* for 25 min. These pellets were resuspended in ice-cold MSH buffer (210 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.2 mM EGTA, 5 mM succinate, 0.15% (w/v) bovine serum albumin (BSA), 5 μM rotenone). The mitochondrial suspensions were then incubated with 10 μM Rh123 for 30 min on ice and washed with ice-cold MSH buffer. Mitochondrial sizes were assayed by the determination of forward light-scatter of suspended mitochondria by flow cytometry.

3. Results

3.1. Influence of anticancer drugs and ^{137}Cs on the susceptibility of OSC-3 cells to LAK cells

OSC-3 cells pretreated with anticancer agents were more sensitive to LAK cells than non-treated cells (Fig. 1a). By the addition of LAK cells (E:T = 10:1), the growth of 5 Gy-irradiated or 1 μg/ml 5-FU or CDDP-pretreated OSC-3 cells was inhibited to approximately 10–30% of the control. Agarose gel electrophoresis revealed the intranucleosomal DNA fragmentation in the OSC-3 cells treated with anticancer agents and LAK cells (Fig. 1b). The DNA fragmentation was increased by the co-cultivation of OSC-3 cells pretreated with anticancer agents with LAK cells. DNA fragmentation was increased by a combination of LAK cells and each anticancer agent (Fig. 1c). The specific [^{3}H]TdR release at 18 h culture was lower than 2% and approximately 7% in OSC cells co-cultured with LAK cells or treated with ^{137}Cs (5 Gy), respectively. However, the specific release was increased to above 13% by irradiation and LAK cells. Likewise, more than additive cooperation was observed in OSC-3 cells pretreated with CDDP and co-cultured with LAK cells. In contrast, 5-FU-treated cells revealed additive DNA fragmentation by co-cultivation with LAK cells. Apoptotic OSC-3 cells were also identified by the terminal transferase-mediated dUTP nick-end labelling (TUNEL) (data not shown).

The increased apoptosis of OSC-3 cells by the combined treatment was also observed in the detection of phosphatidylserine (Fig. 2). The fluorescence intensity was 20.9% in the control cells and the intensity was increased to 30.8% by co-cultivation of OSC-3 cells with LAK cells. CDDP, 5-FU and ^{137}Cs further increased the externalisation of phosphatidylserine (44.4%, 43.4% and 39.3%, respectively), and additive increases of the externalisation were observed in the combinations of LAK cells and anticancer agents.

3.2. Involvement of granzyme B and Fas antigen in DNA fragmentation by LAK cells and anticancer agents

By treatment with ^{137}Cs, 5-FU and CDDP, the expression of Fas antigen on OSC-3 cells was increased approximately 2-fold; in the control, the mean fluorescence intensity was 7.16, but was increased to 16.84, 15.23 and 16.64 following treatment of the cells with 5 Gy ^{137}Cs, 0.5 μg/ml 5-FU and 5 μg/ml CDDP, respectively (Fig. 3).

DNA fragmentation was suppressed by αFas-IgG in the pretreated or non-treated OSC-3 cells, with a similar suppression in all treatments (Fig. 4). αFas-IgG decreased specific [^{3}H]TdR release approximately 25–33% compared with the control levels, while granzyme B inhibitor Z-AAD-CMK also suppressed the [^{3}H]TdR release compared with the control level and caspase-3 inhibitor Ac-DEVD-CHO suppressed the release to approximately half of the control.

3.3. Caspase-3 activation by LAK cells and anticancer agents

Procaspase-3 was weakly cleaved by the anticancer agents and largely cleaved by LAK cells (Fig. 5). In

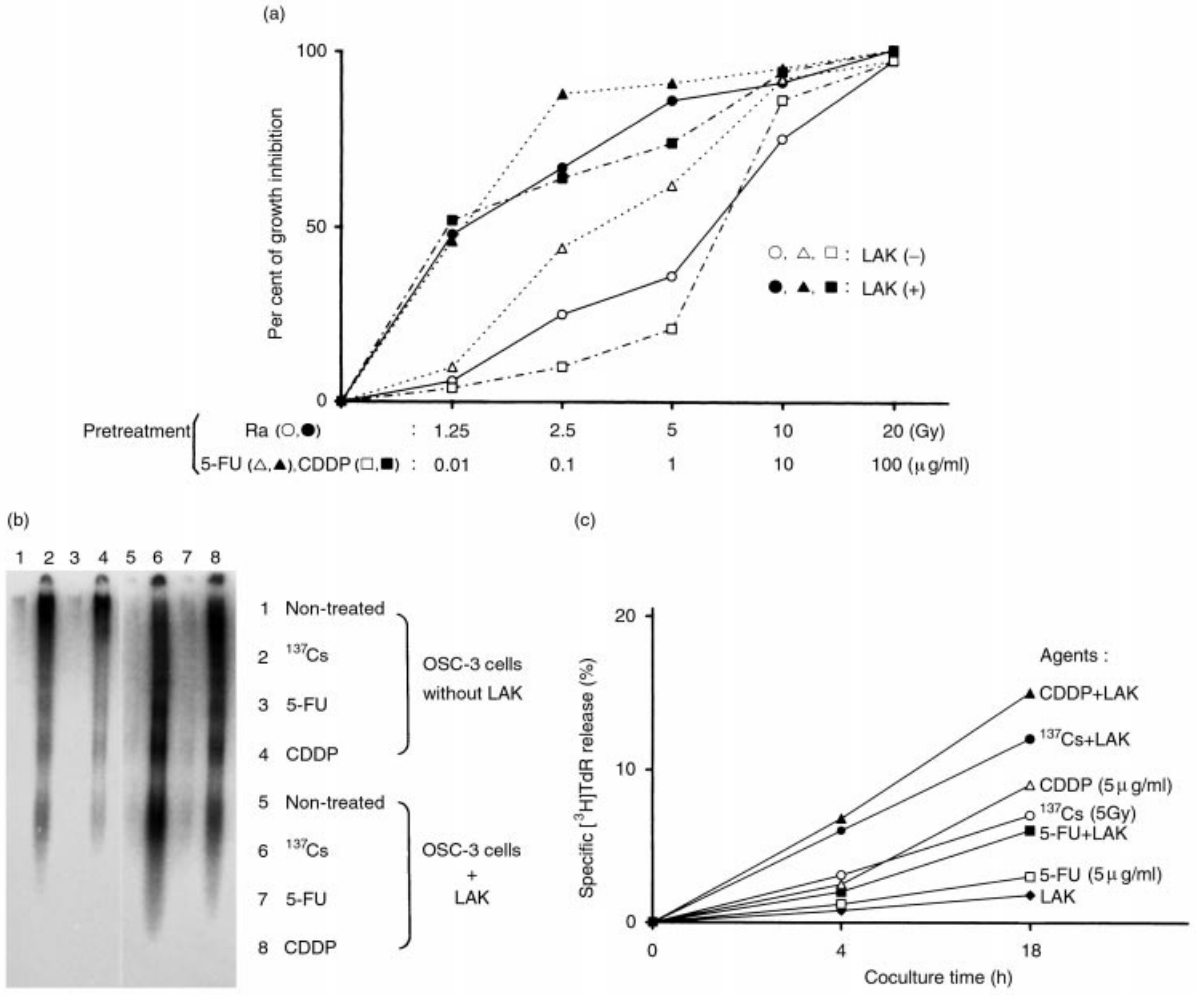

Fig. 1. Influence of anticancer drugs and ^{137}Cs on growth inhibition and DNA fragmentation of OSC-3 cells by LAK cells. (a) Cell growth inhibition assay. OSC-3 cells were irradiated with ^{137}Cs or cultured with the indicated anticancer drugs as previously described. The cells were then co-cultured with LAK cells (E/T = 10:1) for 48 h and the viable cells were counted. (b) Agarose gel electrophoresis: [^{125}I]IdUrd-labelled OSC-3 cells were treated as previously described and co-cultured with LAK cells (E/T = 10:1) for 48 h. The isolated DNA was electrophoresed in an agarose gel and visualised by autoradiography. (c) DNA fragmentation assay. [^{3}H]TdR-labelled OSC-3 cells were treated as previously described and co-cultured with LAK cells (E/T = 10:1) for the indicated times. The cells were then disrupted and released [^{3}H]TdR was measured. In (a) and (c), each experiment was performed in triplicate and all standard deviations were less than 10% of the mean.

parallel with the cleavage, the caspase-3 activities in OCS-3 cells pretreated with anticancer agent were increased to approximately twice the control level by the addition of LAK cells and a small increase was also observed in the untreated cells following the addition of LAK cells.

3.4. Enhanced cleavage of PARP and NuMA by a combination of anticancer agents and/or LAK cells

Being co-ordinated with the caspase-3 activation, cleavage of NuMA from 200 kDA to 175 kDa was enhanced by a combination of the anticancer agents and LAK cells (Fig. 6). OSC-3 cells pretreated with αFas-IgM, ^{137}Cs, 5-FU or CDDP±LAK cells (with the exception of the untreated ^{137}Cs cells) revealed cleavage of PARP from 116 to 85 kDa.

3.5. Cleavage of bid in OSC-3 cells pretreated with anticancer agents and co-cultured with LAK cells

Compared with αFas-IgM, 5-FU treatment resulted in a greater amount of cleaved bid (Fig. 7). In OSC-3 cells co-cultured with LAK cells, the cleaved products of bid were observed, and cleavage of bid was increased by pretreatment with ^{137}Cs or CDDP.

3.6. Intracellular ROI levels in OSC-3 cells pretreated with the anticancer agents and co-cultured with LAK cells

By co-cultivation of OSC-3 cells with LAK cells, the intracellular ROI level was increased to nearly twice the control level (Fig. 8). The increased ROI level was largely decreased by αFas-IgG and Z-AAD-CMK, but was not influenced by Ac-DEVD-CHO. Although ^{137}Cs, 5-FU

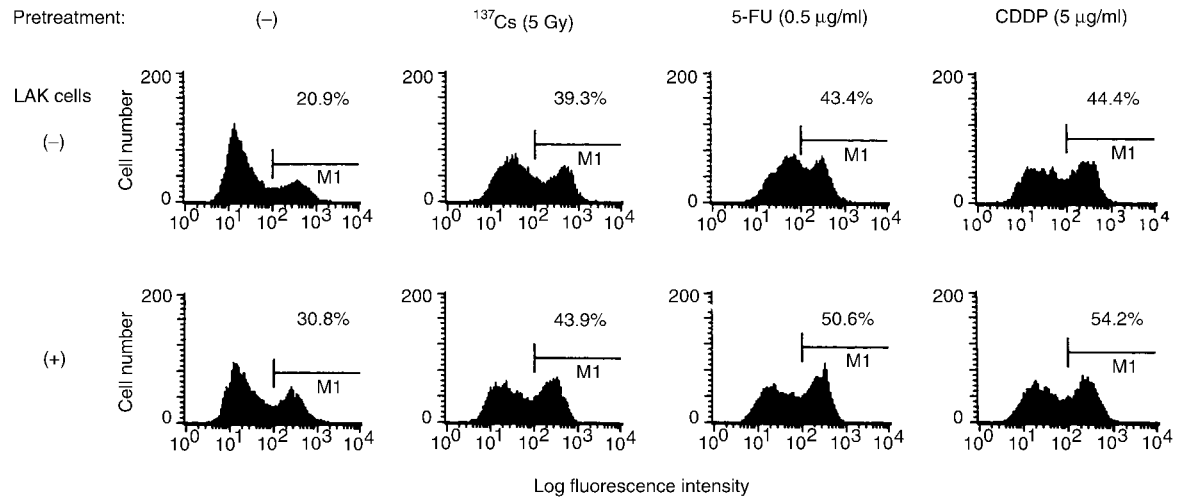

Fig. 2. Enhanced phosphatidylserine externalisation by a combination of anticancer agents and LAK cells. After pretreatment as in Fig. 1(a), OSC-3 cells were co-cultured with LAK cells (E/T = 5:1) for 6 h and the phosphatidylserine level was measured by flow cytometry. The experiment was repeated three times and this is a representative example.

and CDDP did not increase the intracellular ROI level, the combination of LAK cells with 5-FU or CDDP increased the ROI level to at least those levels seen with LAK alone.

3.7. Mitochondrial membrane potential in OSC-3 cells pretreated with anticancer agents and co-cultured with LAK cells

When OSC-3 cells were treated with ^{137}Cs, 5-FU or CDDP, the ψ_m decreased although the mitochondrial size was almost constant (Fig. 9). The decrease of ψ_m was increased by co-cultivation of OSC-3 cells with LAK cells. For example, the mean ψ_m was 20.22 in the non-treated cells and the potential was decreased to 13.52 by 5-FU treatment and to 12.67 by treatment with 5-FU and LAK cells.

4. Discussion

Apoptosis of solid tumour cells is inducible by a variety of anticancer drugs, γ-rays and cytotoxic lymphocytes [9–19]. The mechanism of apoptosis induced by these agents has been elucidated gradually and their apoptotic signal pathways have been clarified. However,

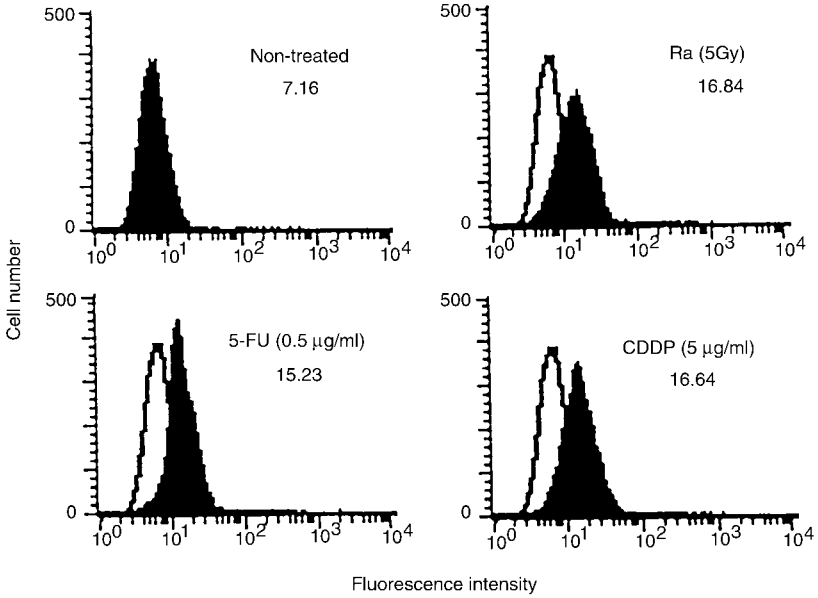

Fig. 3. Influence of anticancer agents on the expression of Fas antigen on OSC-3 cells. OSC-3 cells were irradiated with ^{137}Cs (5 Gy) and then cultured for 48 h in the absence of any anticancer drugs or OSC-3 cells were cultured with the indicated anticancer drugs for 48 h. The expression of Fas antigen on the cells was then measured.

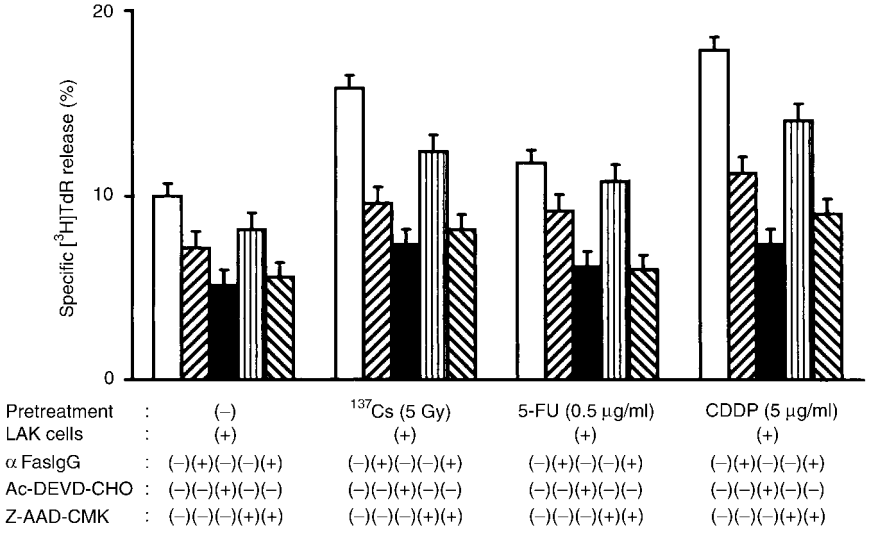

Fig. 4. Involvement of Fas antigen, granzyme B and caspase-3 in DNA fragmentation by LAK cells and anticancer agents. After pretreatment as in Fig. 1(a), [^{3}H]TdR-labelled OSC-3 cells were co-cultured with LAK cells (E:T = 10:1) in the presence or absence of αFas-IgG (500 ng/ml), Ac-DEVD-CHO (50 μM) or Z-AAD-CMK (50 μM) for 18 h and specific [^{3}H]TdR release was measured.

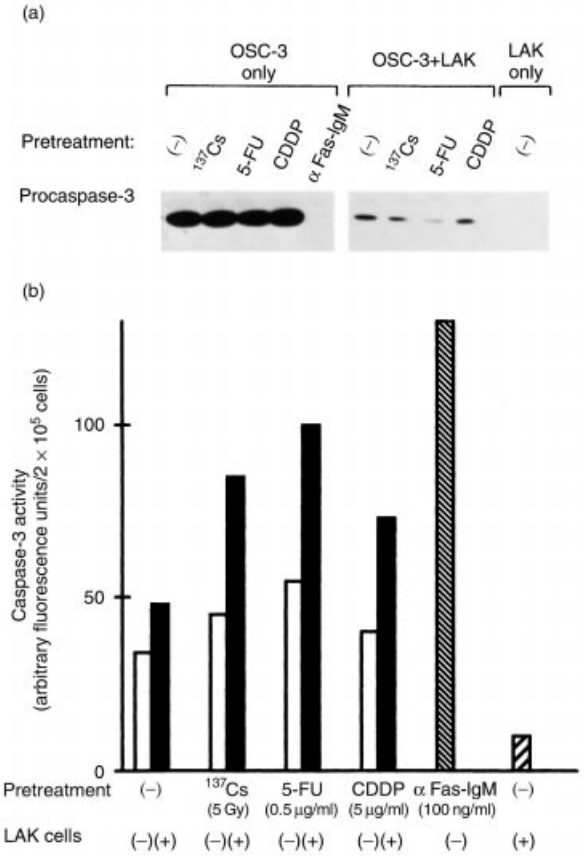

Fig. 5. Caspase-3 activation by LAK cells and anticancer agents. After pretreatment with each anticancer agent or αFas-IgM (100 ng/ml) for 48 h, OSC-3 cells were co-cultured with or without LAK cells (E/T = 5:1) for 18 h. The cells were then disrupted and the cytosolic proteins were subjected to Western blotting analysis (a) and caspase-3 activity assay (b).

details of the pathways remain to be explored, especially in squamous cell carcinoma. It was recently reported that in squamous cell carcinomas, a combination of adoptive LAK therapy with chemoradiotherapy yielded better clinical and histopathological effects than chemoradiotherapy alone [8]. The report indicates the possibility that the combined therapy induced a high level of apoptosis in the tumour cells.

The proliferation of OSC-3 cells was strongly inhibited by co-cultivation with LAK cells after pretreatment with 5-FU, CDDP or γ-rays, and DNA fragmentation of OSC-3 cells was also induced by the combination of these agents and LAK cells. These results represent a modification of OSC-3 cells by these agents to be sensitive to LAK cells. It is well known that the Fas/Fas-L system is involved in the induction of apoptosis by cytotoxic lymphocytes [16,17]. Therefore, the increased expression of Fas on ^{137}Cs-, 5-FU- and CDDP-treated OSC-3 cells appears to support the enhanced DNA fragmentation by the combined treatment. The involvement of Fas in LAK cell-induced apoptosis was also observed in the cleavage of procaspase-8 (data not shown) and the inhibition tests using αFas-IgG. However, as described below, LAK cell-induced apoptosis does not completely depend upon Fas.

Besides Fas, Fas-L was also induced on OSC-3 cells by 5-FU, CDDP and ^{137}Cs (data not shown). Fas-L on tumour cells may induce the apoptosis of Fas-expressing cytotoxic lymphocytes, which is a mechanism of tumour cell resistance to cytotoxic lymphocytes [36–38]. However, Fas-L-expressing OSC-3 cells did not impair LAK cells (data not shown). It was recently reported that cytotoxic lymphocytes possess FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory pro-

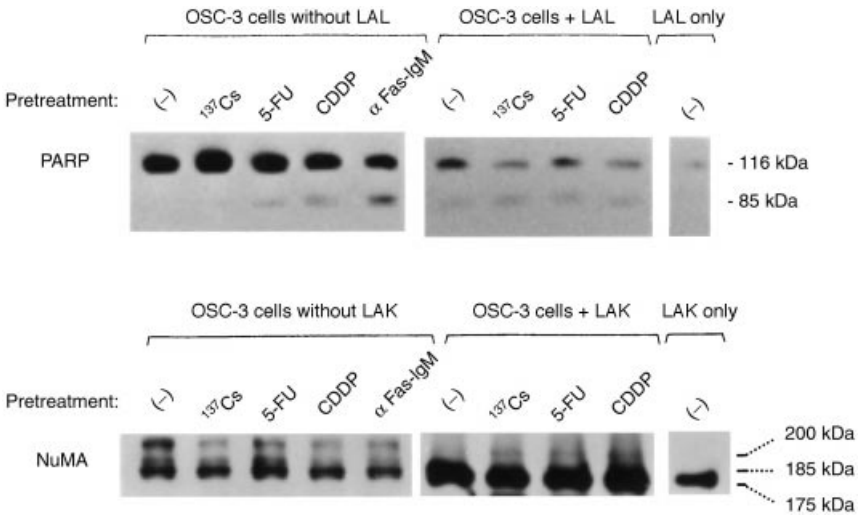

Fig. 6. Enhanced cleavage of PARP and NuMA by a combination of anticancer agents and LAK cells. After pretreatment with each anticancer agent or αFas-IgM (100 ng/ml) for 48 h, OSC-3 cells were co-cultured with or without LAK cells (E:T = 5:1) for 18 h. The cells were then disrupted and the cellular proteins were subjected to Western blotting analysis. The experiment was repeated three times and this is a representative example.

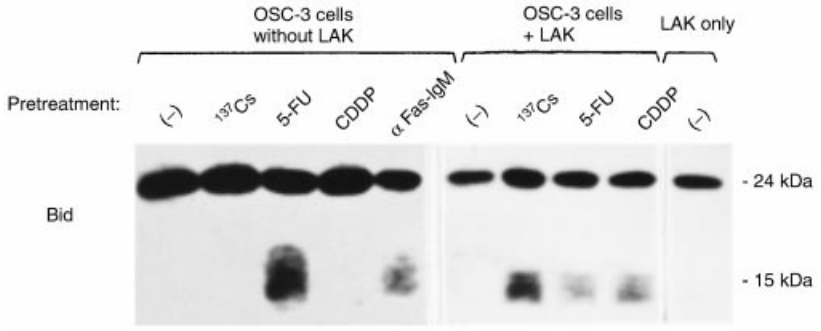

Fig. 7. Cleavage of bid in OSC-3 cells pretreated with anticancer agents and co-cultured with LAK cells. After pretreatment with each anticancer agent or αFas-IgM (100 ng/ml) for 48 h, OSC-3 cells were co-cultured with or without LAK cells (E:T = 5:1) for 18 h. The cells were then disrupted and the cellular proteins were subjected to Western blotting analysis.

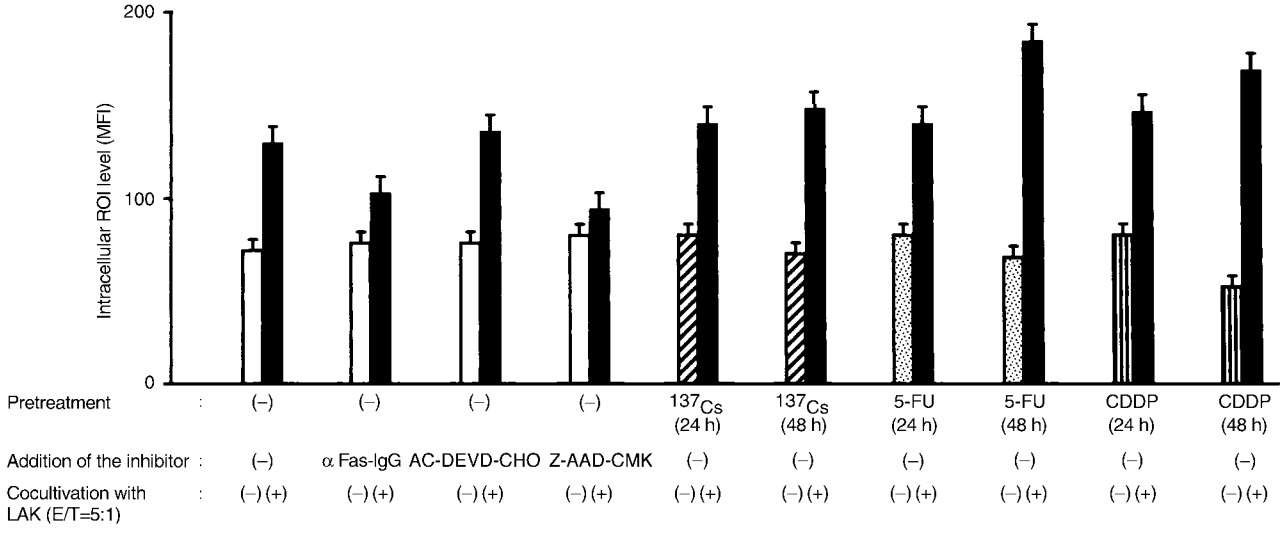

Fig. 8. Intracellular ROI levels in OSC-3 cells pretreated with anticancer agents and co-cultured with LAK cells. After the indicated pretreatment and co-cultivation with LAK cells in the presence or absence of each inhibitor, the cells were labelled with hydroethidine and intracellular ROI levels were measured.

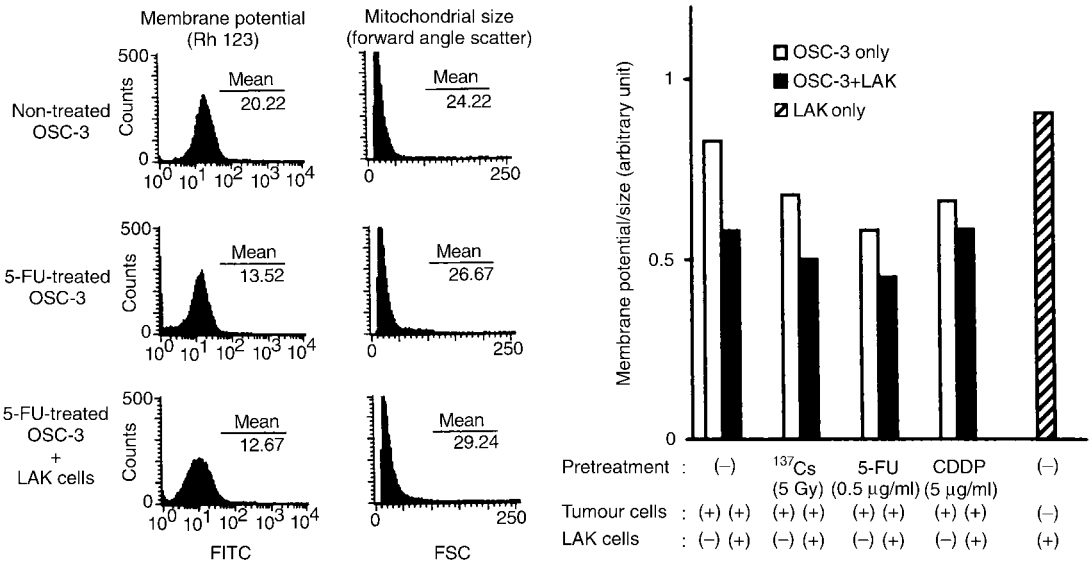

Fig. 9. Mitochondrial membrane potential in OSC-3 cells pretreated with anticancer agents and co-cultured with LAK cells. After pretreatment and co-cultivation (E:T = 5:1) as in Fig. 1(a), mitochondria were isolated from OSC-3 cells. The mitochondrial membrane potentials and sizes were measured as previously described. The experiment was repeated three times and this is a representative example.

tein (FLIP) and protect themselves from Fas-mediated cytolysis through FLIP [39,40]. LAK cells may also have such a protection system.

Externalisation of phosphatidylserine, which is the initial phase of apoptosis [41,42], was enhanced by co-cultivation of pretreated OSC-3 cells with LAK cells. The upregulated expression is compatible with the increased expression of Fas antigen induced by the LAK cells. However, the level of externalisation was not closely correlated with the level of DNA fragmentation. This discrepancy suggests that externalisation of phosphatidylserine does not inevitably lead to DNA fragmentation.

In the execution phase of apoptosis, caspase-3 is activated, and activated caspase-3 degrades multiple proteins such as PARP, NuMA, lamins and protein kinase C delta (δ); [23–25]. Caspase-3 activity was strongly augmented by co-cultivation of OSC-3 cells with LAK cells after treatment with 5-FU, CDDP and ^{137}Cs. The increased caspase-3 activity was compatible with the increased degradation of PARP and NuMA by LAK cells. The LAK cell-induced upregulation of caspase-3 activity was partially suppressed by αFas-IgG and granzyme B inhibitor, Z-AAD-CMK and almost completely suppressed by both reagents (data not shown). Correspondingly, DNA fragmentation induced by LAK cells and the combination of LAK cells and each anticancer agent was reduced by αFas-IgG and Z-AAD-CMK to approximately two-thirds to one half of the control (Fig. 4). Therefore, LAK cell-induced apoptosis depends on both the Fas and granzyme B-mediated pathways, and the contributions of Fas and granzyme B appear almost equal. Additionally, caspase-3 inhibitor, Ac-DEVD-CHO, suppressed DNA fragmentation to approximately half that of the control. This means that approximately 50% LAK cell- and the anticancer agent-induced apoptosis may be mediated by caspase-3.

It has been reported that granzyme B induces caspase-independent apoptosis by the direct proteolysis of death substrates [19]. DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}) and NuMA are directly cleaved by granzyme B. Although these substrates can be also cleaved by caspase-3, the fragments cleaved by caspase-3 and granzyme B are distinct. Granzyme B degrades NuMA to a 175 kDa fragment, which is distinct from a 185 kDa fragment by caspase-3. In the present examination, NuMA was cleaved to a 185 kDa fragment in the ^{137}Cs-, 5-FU- or CDDP-treated OSC-3 cells and to a 175 kDa fragment in OSC-3 cells co-cultured with the LAK cells. Therefore, it is likely that granzyme B released from LAK cells directly cleaves NuMA, but that the anticancer agents activate caspase-3 and it is the activated caspase-3 that cleaves NuMA.

Recently, the role of bid in apoptotic signal transduction has been studied and mediation of the signal from caspase-8 to the mitochondria clarified [43–46]. The present study revealed activation of bid by αFas-IgM and 5-FU (Fig. 7). Based on the recent investigation, bid activation by αFas-IgM seems to be mediated by caspase-8. However, the molecules in the bid cleavage by 5-FU are obscure. To clarify the molecules, we examined whether bid was activated by other caspases, but we could not detect bid activation by caspases except for caspase-8. Additionally, the enhanced bid activation by these agents was not lowered by the caspase-3 inhibitor (data not shown). Therefore, bid activation by a downstream or upstream signal is unlikely. Bid amplifies apoptosis-inducing signals by stimulating

mitochondrial ROI generation via a bid-associating protein, such as bcl-2 and bax [45]. In LAK treated cells, ^{137}Cs and CDDP pretreated OSC-3 cells, ψ_m was reduced. However, these cells showed no activation of bid. In OSC-3 cells co-cultured with LAK cells, the intracellular ROI level was increased and the increased ROI level was decreased by the addition of αFas-IgG and Z-AAD-CMK to the co-culture system, suggesting the role of granzyme B in the generation of the ROI. In OSC-3 cells treated with ^{137}Cs and CDDP, ROI could potentially play a role in the reduction of ψ_m. Although it is uncertain whether ^{137}Cs and CDDP directly increase the intramitochondrial ROI level or not, the ROI levels in the mitochondria of OSC-3 cells treated with them were increased (data not shown). Therefore, the reduction of ψ_m could result from the increased ROI.

Some investigators have reported the role of ROI in apoptosis [47–49]. However, the precise role of ROI in the pathway of apoptosis, especially in the apoptotic signal by cytotoxic lymphocytes, has not yet been clarified. The present study revealed an increase of intracellular ROI levels in OSC-3 cells which were treated by LAK cells. The partial suppression of the increased ROI levels by αFas-IgG and Z-AAD-CMK suggests that LAK cells induce ROI in the target cells through Fas and granzyme B, but that other mediators may also be involved in this induction.

It is well known that ROI generated in the mitochondria decrease ψ_m [30,31]. In fact, ψ_m was lowered even further by the combined OSC-3 treatment. A decrease of ψ_m leads to the opening of the pore in the outer mitochondrial membrane, resulting in release of cytochrome c. Therefore, the increase of intracellular ROI level by LAK cells and the combination of LAK cells and anticancer agents suggests enhanced cytochrome c release. In squamous cell carcinoma cells treated with LAK cells and anticancer drugs or γ-rays, caspase-9 may be activated. Thus, ROI appear to play an important role in LAK cell-induced apoptosis. However, the details of the apoptotic signal in squamous cell carcinoma cells remains to be explored.

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