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Apoptosis

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INTRODUCTION

Apoptosis is the predominant form of eukaryotic cell death observed in variety of physiological and pathological conditions such as cancer involution, metamorphosis, development of the immune and nervous system, and embryogenesis (Wyllie et al., 1980). It is also induced by cytotoxic T lymphocytes and natural killer cells, by such cytokines as tumor necrosis factors and lymphotoxins, and by glucocorticoids. It had been also reported that the tumoricidal activity of several chemotherapeutic agents in various tumor cells is mediated via endogenous apoptotic pathway. Much of the recent literature on apoptosis cites the landmark paper of Kerr, Wyllie, and Curri published in 1972. The most characteristic morphological findings of apoptosis are segmentation of the nucleus, condensation of the cytoplasm, membrane blebbing and appearance of apoptotic bodies. The best defined biochemical event in apoptosis involves nuclear DNA. Double-strand cleavage of DNA is observed at linker regions between nucleosomes. Those 180-200 base pair fragments are readily shown by agarose gel electrophoresis of DNA and it appears as a typical ladder pattern. Other biochemical features such as the expression of special genes (*p53, bcl-2, myc, etc.*) and the synthesis of specific proteins (endonuclease, transglutaminase, glutathione S-transferase, heat shock proteins and poly (ADP-ribose) phosphorylase, etc.) are observed. But it has been more difficult to define the biochemical findings of apoptosis, partly because apoptosis occurs asynchronously.

In this paper, I describe the significance and the cardinal morphological and biochemical findings of apoptosis in several cultured tumor cells induced by adenosine(Ado), carbocyclic Ado analogues, prostaglandins(PG) and haptoglobin(Hp)-hemoglobin(Hb) complex(Cx).

DIFFERENCES BETWEEN APOPTOSIS AND NECROSIS

Apoptosis is a distinct type of cell death that differs fundamentally from necrosis in its nature and biological significance. Apoptosis is an active process of gene directed cellular self destruction and in most of the circumstances in which it occurs, it serves a biologically meaningful homeostatic function. In contrast, necrosis is essentially accidental in its occurance, being the outcome of severe injurious changes in the enviroment of the affected cells. General differences between apoptosis and necrosis are listed in Table 1.

INDUCTION OF APOPTOSIS

Apoptosis induced by chemotherapeutic agents

A wide variety of physical and chemical stimuli induce apoptosis in various cells. Though the exact selection mechanism of the biochemical pathway of cell death is not fully understood. It is clear that cells possess effector mechanisms for self-destruction, which may be activated by several relatively mild stimuli. As shown in Table 2, a wide spectrum of chemotherapeutic agents induces apoptosis in different cancer cells. It is evident that apoptosis can be

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Table 1. General differences between apoptosis and necrosis.

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological	Pathological (injury)
Occurrence	Single cells	Groups of cells
Reversibility	No (after morphological changes)	Yes (up to the point of no return)
Adhesions between cells and to BM	Lost (early)	Lost (late)
Cytoplasmic organelles	Late stage swelling	Very early swelling
Lysosomal enzyme release	Absent	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrhexis)	Disappearance (karyolysis)
Nuclear chromatin	Compaction in uniformly dense masses	Clumping not sharply defined
DNA breakdown	Internucleosomal	Randomized
Cell	Formation of apoptotic bodies	Swelling and later disintegration
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
Scar formation	Absent	Present

Table 2. Examples of anticancer agents reported to induce apoptosis in cultured cells.

Drug	Cell lines tested
Amsacrine	Thymocytes
Aphidicoline	CHO strain AA8
	CHO strains AA8, UV 41
1-β-D-Arabinofuranosyl-cystosine	HL60, KGIA
BCNU	CCRF/CEM C7, F89, Molt-4-F, EB1, EB2-3945
Camptothecin	HL60, KGIA
Cisplatin	CHO strains AA8, UV 41
	HL60, KGIA
	L1210/0
Etoposide	Thymocytes
	CHO strains, AA8, UV 41
	HL60, KGIA
	CHO
	Chronic lymphocytic leukemia
5-Fluorodeoxyuridine	CHO strains AA8, UV 41
5-Fluorouracil	CHO strains AA8 UV 41
	CCRF/CEM C7, F89, Molt-4-F, EB1,
	F89, Molt-4-F, EB1, EB2-3945
Methotrexate	CHO strains AA8, UV 41
	HL60, KGIA
Melphalan	CCRF/CEM C7, F89, Molt-4-F, FB1, EB2-3945
Teniposide	Thymocytes
Vincristine	CHO strain AA8
	F89, Molt-4-F, EB1, EB2-3945

induced by a variety of drugs with diverse chemical structures and different mechanisms of action.

Among the long list of drugs reported to induce apoptosis are (i) the DNA-damaging agents, such as BCNU, melphalan and *cis*-platinum, known to cause several types of DNA damage, such as DNA interstrand and intrastrand crosslinks and DNA-protein crosslinks; (ii) drugs like camptothecin that cause protein-assocated DNA strand breaks mediated by the enzyme DNA topoisomerase I; (iii) the epipodophyllotoxins and intercalating agents that are poisons of DNA toposomerase II; (iv) inhibitors of mitotic spindle apparatus, such as the vinca alkaloids ; (v) inhibitors of DNA synthesis (aphidicolin); and (vi) several antimetabolites. The list of chemotherapeutic agents inducing apoptosis will continue to expand along with growing interest in this type of cell death, especially in tumor cells, for the development of a new chemotherapeutic agent.

Apoptosis induced by adenosine and carbocyclic adenosine analogues

S-adenosylhomocysteine (AdoHcy) hydrolase catalyzes the reversible cleavage of AdoHcy to Ado and L-homocysteine (Hcy). Ado is one of the well known inhibitor of AdoHcy hydrolase and recently, specific inhibitors of AdoHcy hydrolase have been developed and synthesized. Among them, carbocyclic Ado analogues such as 3-deazaadenosine (DZA), 3-deaza-aristeromycin (DZAri) and 3-deaza-nephlanocin (DZNep) are well known potent competitive inhibitors of AdoHcy hydrolase (Fig. 1). These inhbitors showed a number of interesting biological activities such as antiviral action, inhibition of neutrophil chemotaxis, induction of HL-60 leukemic cell growth, and cytostatic activity on macrophage multiplication.

There has recently been a major focus on the role of apoptosis in the pathogenesis of disease. Severe combined immunodeficiency disease (SCID), an infant disorder, is associated with a deficiency of adenosine deaminase (ADA) and is correlated with defects in both cellular and humoral immunity, resulting in an early death. All SCID patients have elevated tissue levels of Ado and deoxy-Ado. It has been shown that when high concentrations of Ado or inhibitors of ADA are added to cells, the viability and functions of lymphocytes, macrophages, neutrophils and fibroblasts are severely depressed. In addition, AdoHcy, formed by AdoHcy hydrolase from Ado and Hcy, can inhibit methylation reactions in cells. The inhibition of cellular methylation by AdoHcy has also been suggested to be involved in the pathogenesis of SCID. However, the molecular events involved in the apoptotic effect of high dose of exogenous Ado on lymphocytes have not been examined. High concentration of Ado, when added to L1210 lymphocytic leukemia cells, resulted in apoptosis (Kim et al., 1994). The apoptotic process was accompanied by distinct morphological changes of apoptosis. Extensive DNA fragmentation was correlated with Ado concentrations (Fig. 2). Furthermore, apoptosis in these cells was preceded by an early but transient expression of *c-myc* protooncogene (Fig. 3), and was not influenced by Hcy addition Since severe SCID is associated with a deficiency of ADA, leading to defects in both cellular and humoral immunity, Ado-induced apoptosis may thus be a contributing factor in the pathogenesis of SCID.

It has been reported that the tumoricidal activity of several chemotherapeutic agents in various tumor cells is mediated *via* endogenous apoptotic pathway.

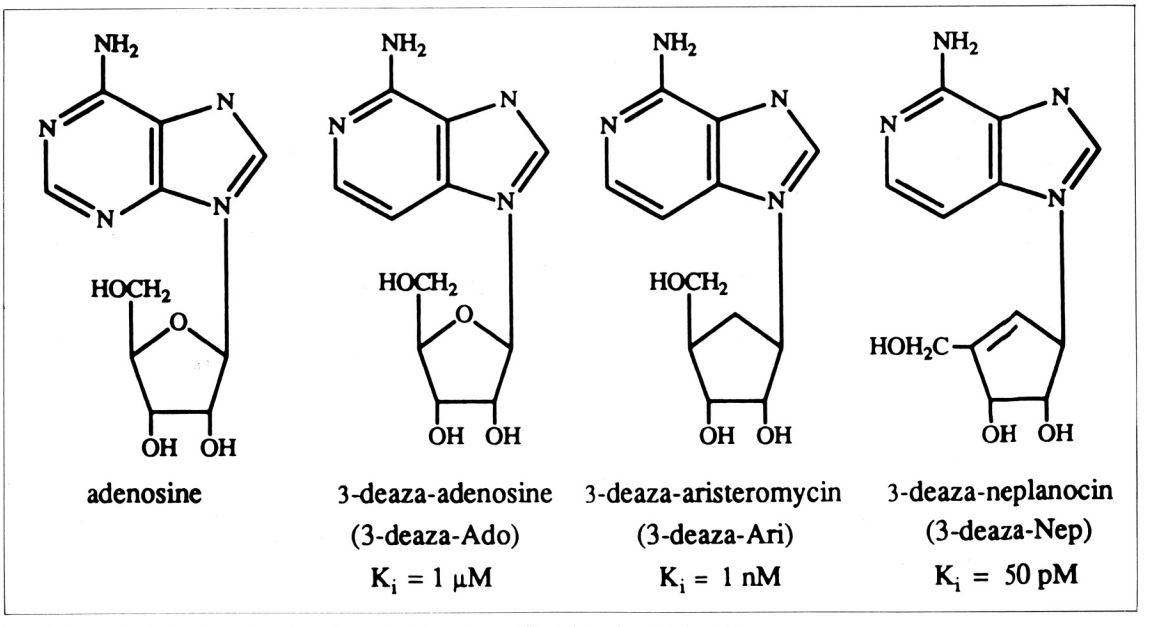

Fig. 1. Chemical structure of adenosine and it's carbocyclic adenosine analogues.

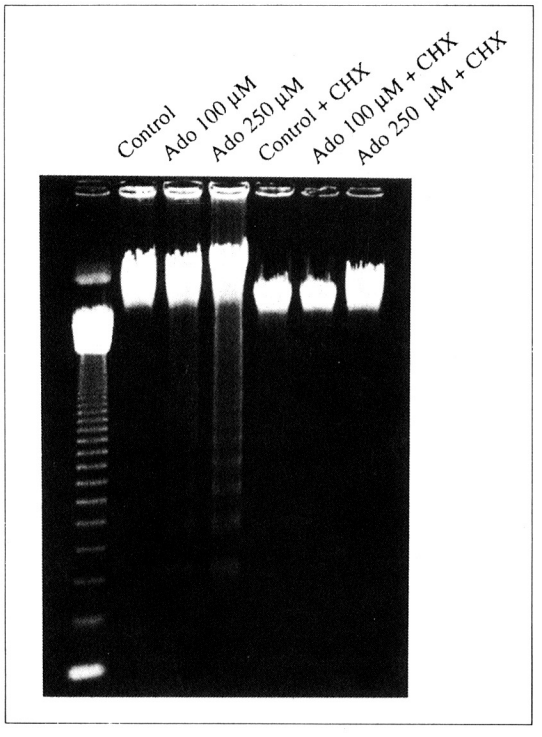

Fig. 2. DNA fragmentation in L1210 cells treated with Ado. Cells were treated as indicated for 72 h. Cycloheximide (CHX; 0.5 μg/ml) was added 30 min prior to Ado treatment. Unlabelled lane=123 bp DNA ladder.

Carbocyclic Ado analogues showed antiproliferative activity on the growth of several tumor cells, and it was also reported that Ado and deoxy-Ado induced apoptosis in thymocyte and mouse leukemic L1210 cells (Kim et al., 1994). These findings implicated that the antiproliferative effects of carbocyclic Ado analogues on the growth of tumor cells is strongly related to the activation of endogenous apoptotic pathway. When L1210 cells were treated with DZA and DZAri resulted in the arrest of G_2/M and G_0/G_1 phase of cell cycles, respectively. These carbocyclic Ado analogues showed characteristic morphological and biochemical changes of apoptosis and cycloheximide (CHX) completely blocked the ladder DNA induced by DZA and DZAri (Fig. 4). These results suggest that DZA and DZAri have antiproliferative effects on the growth of L1210 cells *via* endogenous apoptotic pathway, and protein of which synthesis is stimulated by carbocyclic Ado analogues may be deeply involved in the initiation or progression of this apoptosis

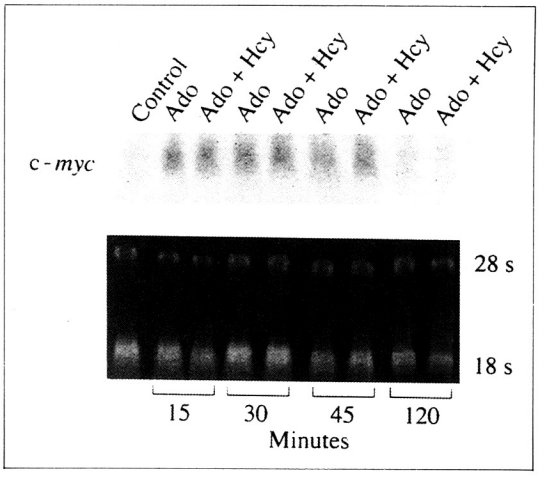

Fig. 3. Northern blot analysis of the expression of proto-oncogene *c-myc* during the initiation of apoptosis in L1210 cells treated with 250 μM Ado with or without 100 μM Hcy.

(Lee et al., 1995). The nature of the protein is under.

Apoptosis Induced by PGA_2 and Δ^{12}-PGJ_2

Cyclopententenone PGs such as PGA_2 and Δ^{12}-PGJ_2 are known to be the enzymatic dehydration products PGE_2 and PGD_2 respectively, have potent antiproliferative activities on the growth of various cultured tumor cells. It has been shown that PGA_2 and Δ^{12}-PGJ_2 are actively transported to nuclei and bind to nuclear proteins, and the binding of the PG molecule to nuclear proteins appears to regulate the expression of specific genes which are responsible for tumor cell growth inhibition. But the precise molecular mechanism by which these PGs exert cytostatic or cytotoxic activity on the tumor cell growth remains unclarified.

Treatment of L1210 cells with PGA_2 and Δ^{12}-PGJ_2 resulted in significant G_2/M arrest and revealed typical morphological and biochemical changes of apoptosis. CHX blocked the DNA fragmentation and morphological changes induced by Δ^{12}-PGJ_2 (Fig. 5).

Human hepatocarcinoma cells (SK-HEP-1) appeared to die through apoptosis with characteristic morphological and biochemical changes of apoptosis by treatment with Δ^{12}-PGJ_2. The induction of apoptosis by Δ^{12}-PGJ_2 in SK-HEP-1 cell was also blocked by CHX. Western blot analysis using anti-p53 and anti-WAF1 monoclonal antibodies demonstrated that these two protein levels were increased 3 hours after

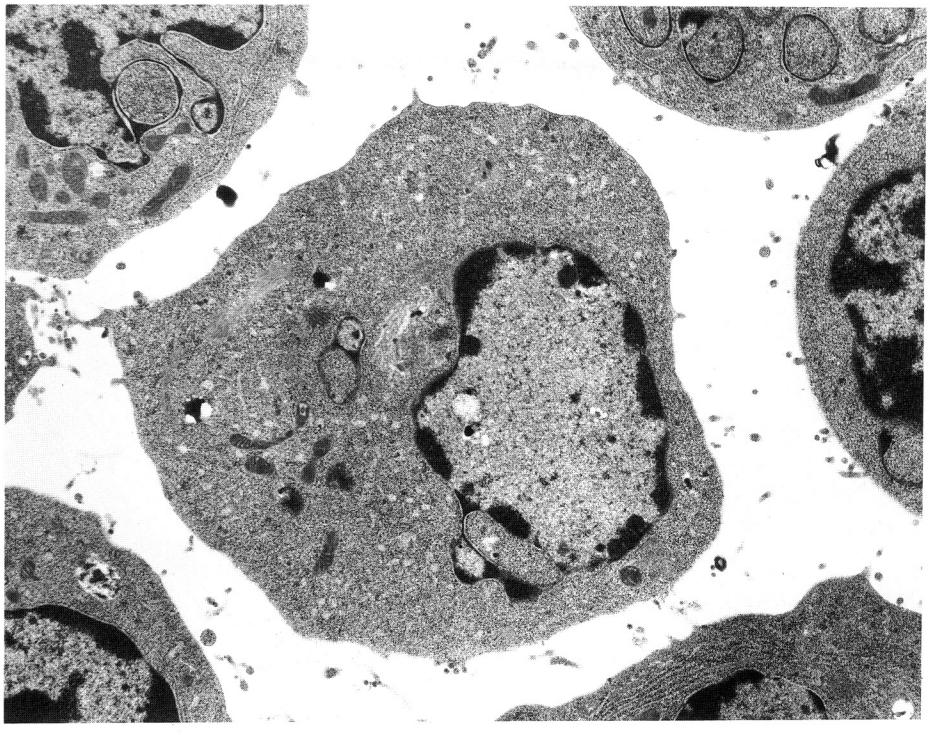

Fig. 4. Electron micrscopical appearance of L1210 cells treated with 100 μM of 3-deazaadenosine for 24 h. (magnification 10,800×).

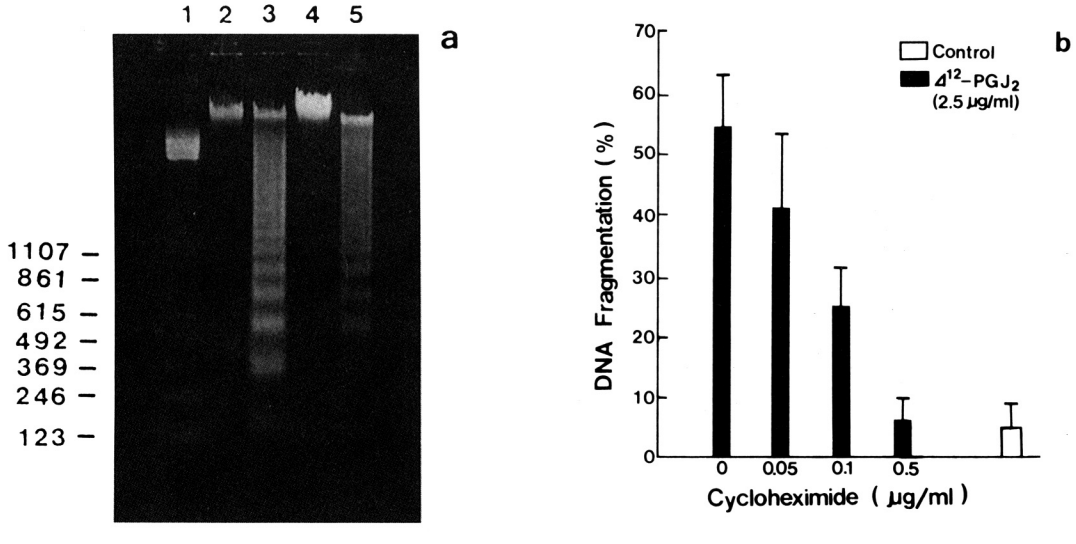

Fig. 5. Inhibition of DNA fragmentation by cycloheximide (CHX). (A) L1210 cells were preincubated for 1 h without pretreatment (lane 3), with 0.5 μg/ml of CHX(lane 4) or 2 ng/ml of actinomycin-D (lane 5). After 48 h of additional incubation with 2.5 μg/ml of Δ^{12}-PGJ_2, cells were lysed and DNA was analyzed gel electro-phoresis. Lane 1 is marker and lane 2 is DNA from control cells. (B) Cells were pretreated with various concentrations of CHX for 1 h and further incubated for 48 h in the presence of 2.5 μg /ml of Δ^{12}-PGJ_2. DNA fragmentation was quantitatively determined using diphenylamine reagent.

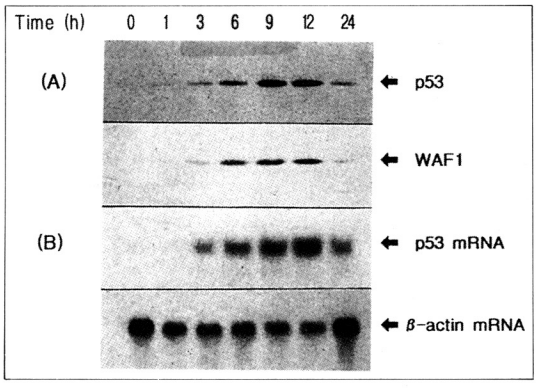

Fig. 6. Levels of p53 and WAF1 proteins (A) and p53 mRNA (B) following Δ^{12}- PGJ_2 treatment. (A) Immunoblot analysis of p53 and WAF1 proteins. Total cellular proteins from 1×10^5 cells were treated with 5 μg/ml of Δ^{12}-PGJ_2 for the indicated times, and subjected to SDS-PAGE at the indicated times after exposure to 5 μg/ml of Δ^{12}-PGJ_2. p53 bands were revealed by an indirect immu-noperoxidase method using enhanced chemiluminescence detection reagents. (B) Northern blot analysis of p53 mRNA. RNA was isolated from each preparation and electrophoresed through a fromaldehyde agarose gel. The transferred RNA was hybridized with DIG-labeled probes for p53 and β-actin and processed for detection.

Δ^{12}-PGJ_2 treatment, and accumulated for up to 12 hours. The induction of p53 protein seemed to be dependent on the increase of p53 mRNA level (Fig. 6). These findings suggest that the cyclopentenone PGs causes the apoptotic cell death of L1210 and SK-HEP-1 cells which is preceded by G_2/M accumulation and requires *de novo* protein synthesis, and that the apoptosis in SK-HEP-1 cells involves a certain process required for p53 induction (Kim et al., 1993; Lee et al., 1995).

Haptoglobin-hemoglobin complex Induces Apoptosis

Hp is an α_2-glycoprotein in serum which binds Hb stoichiometrically *in vitro* and *in vivo*. The chemical nature of Hp is well documented. Although such physiological functions of Hp as prevention of loss of Hb-iron through the kidney in case of hemoglobinemia, inhibition of cathepsin B activity, bacteriostatic action and various other functions have been suggested, but the exact biological function of Hp is still uncertain. The Hp level of plasma is increased in inflammation and malignant neoplasia (Owen et al., 1964; Lee et al., 1995). Possible anti-inflammatory significance of the increased Hp level in inflammatory state was suggested such as the inhibition prostaglandin synthetase activity (Shim, 1976). Likewise, the increased level of Hp in malignancies would have some biological implications. Recently, it was reported that Hp-Hb Cx exhibited antitumor effects against murine $P388D_1$, L1210 and rat RBL tumor cell lines *in vitro*, but non-tumorous RAW264 macrophage and NIH3T3 fibroblast cells were not affected by the Cx (Shim et al., 1985). However, the precise antiproliferative mechanism of Hp-Hb Cx on the growth of tumor cells is still unknown.

Cx showed a dose dependent cytotoxic effect on the growth of Hep3B (human hepatocarcinoma) cells. The antiproliferative effect of the Cx on the multiplication of Hep3B cells was augmented by PGD_2. Anti-human Hb IgG abolishes the effect of the Cx dose dependently (Fig. 7) which indicates that the Cx really exerts the antiproliferative effect. Hep3B cells treated with the Cx showed characteristic biochemical changes of apoptosis such as the DNA fragmentation which was blocked by pretreatment with CHX and the

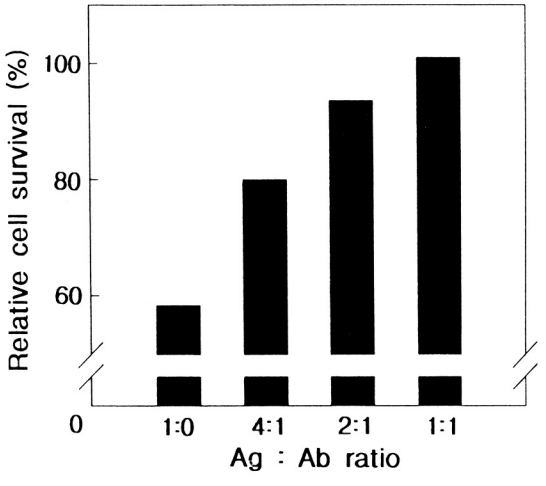

Fig. 7 Effect of rabbit anti-human Hb IgG on the anti-tumor activity of Hp-Hb Cx in Hep3B cells. Equivalent amount of antibody to precipitate Cx completely in 100 μl of 1mg/ml of Cx solution (Ag : Ab ratio=1 : 1) or the same amount of serially two fold diluted antibody solutions were mixed with 100 μl of 1 mg/ml of Cx. After immunoprecipitate was removed, the supernatant was added to the culture media. The cells (5×10^3/100 μl in 96 well plate) were incubated for 48 hours. Cell viability was determined by the MTT method. Data represents the mean of duplicate experiments.

increment of transglutaminase expression. These results suggest that the antiproliferative effect of the Cx against Hep3B cells occurs *via* the typical apoptotic pathway (Kim et al., 1995).

CONCLUSION

We have been studying the apoptotic mechanism induced by PGs and carbocyclic Ado analogues since 1985. The characteristic biochemical and morphological changes of apoptosis were induced in several tumor cell lines by the treatment of Ado, carbocyclic Ado analogues, PGs and Hp-Hb Cx. These findings implicate that the antiproliferative effects of carbocyclic Ado analogues and PGs on the growth of tumor cells are mediated *via* endogenous apoptotic pathway. One particularly interesting feature of apoptosis is its prevention by inhibitors of protein synthesis. However, CHX does not suppress apoptosis in all circumstances. When tumor cells were pretreated with CHX before the addition of each apoptosis inducing agent, the cardinal morphological and biochemical findings of apoptosis are completely blocked. These findings suggest that the apoptosis induced by PGs and carbocyclic Ado analogues require *de novo* protein synthesis. Biochemical characteristics and physiological roles of the genes and the proteins of which expression and syntheses are stimulated by PGs and carbocyclic Ado analogues are under investigation.

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