Possible involvement of glucocorticoid hormones in aflatoxin B₁ hepatocarcinogenesis as a tumor promoter

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It is widely known that aflatoxin B₁ (AFB₁) is one of the environmental risk factors for human liver cancer especially in South-East Asia, China and sub-Saharan Africa¹⁻³. Molecular epidemiological approaches show the high frequency of mutation in codon 249 of the tumor suppressor p53 in these endemic areas^{4,5}. Over expression of *c-myc* gene⁶ and point mutation of *ras* family oncogenes^{7,8} have been reported in AFB₁-induced rat hepatocellular carcinomas. However, the detailed mechanism of AFB₁ hepatocarcinogenesis is still unknown.

Apoptosis is a genetically encoded cell death program defined by characteristic morphology and biochemical changes, and is regulated by various signals from both intracellular and extracellular milieu⁹. Apoptosis has a role in many aspects of normal development, maintaining homeostasis and a number of diseases including cancer, autoimmune and neurodegenerative diseases. In carcinogenesis, development of malignancy has been suggested to result not only from abnormal cell proliferation but also from decreased apoptosis^{10,11}. Now, anticancer agents have been recognized to kill tumor cells through apoptosis¹². Thus, the elucidation of apoptotic cell death mechanism is a critical step for the understanding of carcinogenesis.

It is well established that endocrine factors play an important role in carcinogenesis. Hypophysectomy causes the downregulation of synthesis of steroid hormones and protects against hepatocarcinogenic action of AFB₁ in the rat due to a change of metabolism of AFB₁¹³ or to a reduced uptake of AFB₁¹³. Hepatocyte proliferation *in vivo* and *in vitro* is stimulated by 17 β -estradiol (E₂), while tamoxifen (TAM), an estrogen antagonist, inhibits DNA synthesis in hepatocytes¹⁵. TAM and its derivatives antagonize the interaction between estrogen receptor and its coactivator GRIP1 through the binding to the ligand-binding domain of the receptor¹⁶. Recently, TAM has been reported to induce apoptosis in ER-negative breast cancer MDA-231 cells by the induction of *c-myc* gene expression¹⁷.

It has been shown that TAM induces the secretion of transforming growth factor β 1 (TGF- β 1) in human breast cancer cells and fibroblast, in which TGF- β 1 acts as a growth-inhibitor. TGF- β 1 belongs to a family of 25 kDa dimeric and multifunctional proteins including the regulation of cell proliferation, differentiation and morphogenesis¹⁸. TGF- β 1 plays an important role in terminating liver regeneration and induces apoptosis in hepatocytes and hepatoma cells^{19,20}. In colon cancer cells, point mutation of type II TGF- β receptor gene has been reported²¹. Thus, loss of response to TGF- β associates with the tumor development and progression.

Previously we demonstrated that in Reuber hepatoma H4-II-E cells, metabolically activated AFB₁ interferes with the induction of tyrosine aminotransferase, a liver specific enzyme, through

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the inhibition of glucocorticoid (GC) receptor-hormone complex formation²²). We also found that dexamethasone (DEX), a synthetic GC hormone, stimulates the colony formation of AFB₁-induced rat hepatocellular carcinoma Kagura-2 (K2) cells in semi-solid medium²³, and prevents apoptotic K2 cell death evoked by sodium 5,6-benzylideneascorbate (SBA), which is a benzylaldehyde derivative and possesses an anticancer activity²⁴). Benzylaldehyde has been identified as the main active substance in extracts of the fig fruit (*Ficus carica* L) used traditionally in the treatment of cancer. Benzylaldehyde is reactive compound, which is easily oxidizable and induces strong local irritation²⁵). These results suggest the possibility that GCs play a critical role in AFB₁ hepatocarcinogenesis through the suppression of arachidonic acid (AA) cascade, since DEX is well known to inhibit phospholipase A₂ (PLA₂) activity²⁶).

AA is released from *sn*-2 position of the glycerol in phospholipids by PLA₂ to initiate the syntheses of prostaglandins (PGs) and leukotrienes (LTs) by the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways, respectively. These AA metabolites regulate cell growth and differentiation, immune response, inflammation, and viral replication. In addition to these functions, PGs and LTs have been reported recently to regulate cell survival and apoptosis²⁷⁻²⁹).

On the basis of these reports, we investigated K2 cell death triggered by TAM and TGF- β 1 to understand the implication of endocrine factors in AFB₁ hepatocarcinogenesis. As described below, the obtained results show that AA metabolites are involved in apoptotic K2 cell death elicited by TAM and TGF- β 1, and GCs could act as a tumor promoter in AFB₁ hepatocarcinogenesis through the prevention of apoptosis induced by AA metabolites formed *in vivo*³⁰).

TAM induce apoptotic cell death

We examined the cytotoxic effect of TAM on K2 cells by the trypan blue exclusion method. In the presence of 5×10^{-6} M TAM, the fraction of viable cells was decreased in a time-dependent manner and reached at 60% after 48 hr. Cell viability was also dose-dependently depressed by the continuous treatment of 10^{-6} – 10^{-5} M TAM for 48 hr, and the maximal depression observed at 1×10^{-5} M TAM was estimated to be $37 \pm 3\%$ of viability.

To examine whether the cell death induced by TAM is apoptosis, we analyzed DNA fragmentation profile in the cells treated with 5×10^{-6} M TAM for various times. A typical DNA of oligonucleosomal fragments was detectable within 36 hr after the treatment. This result was further confirmed by a flow cytometric analysis. Nontreated control K2 cells showed a typical pattern of DNA contents reflecting G₀/G₁, S and G₂/M phases of the cell cycle. In contrast, the fraction containing the reduced level of DNA was observed in K2 cells treated with 5×10^{-6} M TAM for 48 hr due to the generation of apoptotic bodies, and the fraction of S phase was reduced from 28.1% to 10.2% when compared with the control.

Moreover, time course study with histogram of PI fluorescence versus light scatter, which is established as one of the methods of assessment of cell cycle dependent apoptosis based on the apoptotic cells having less light scatter than those of viable cells, was performed. The degrees of light scatter of some of G₁ and S phase cells at 12 and 30 hr after the treatment of 5×10^{-6} M TAM, were decrease, while those of G₂/M phase cells were not changed. This is a typical pattern resulted from apoptosis induced the cells existing in G₁ and S phases, showing that TAM

might preferentially induce apoptosis in G₁ and/or S phase of K2 cells.

Inhibition of TAM-Induced apoptosis by GC hormones, but not E₂

We have reported that synthetic GCs such as DEX and TA markedly stimulate the growth of K2 cells in semi-solid and serum-free medium²³⁾ and suppress apoptotic K2 cell death induced by SBA²⁴⁾. We therefore investigated the effect of various steroid compounds on TAM induced apoptosis in order to elucidate the apoptotic induction mechanism. K2 cells were treated with 5×10^{-6} M TAM in the presence of 1×10^{-6} M steroid compounds for 48 hr. Authentic and synthetic GCs efficiently suppressed the cell death compared with other steroid compounds including E₂. On the other hand, the cytotoxic activity of TAM was stimulated in the presence of *trans*-androsterone or (+)-4-androstene-13, 17-dione. The reason is presently unclear. DNA fragmentation observed in TAM-treated K2 cells also disappeared in the presence of 1×10^{-6} M GCs, while other steroid compounds did not show any effects.

Implication of COX pathway in TAM-induced apoptosis

One of the multiple functions of GCs is the suppression of cytoplasmic PLA₂ (cPLA₂) activity²⁶⁾. Activation of cPLA₂ permits the release of AA from membrane-bound phospholipids, which is implicated in many signal transduction pathways including the cell death signal of TNF^{31,32)}. We therefore investigated the effects of COX and LOX inhibitors on TAM-induced apoptosis, to examine whether AA metabolites are implicated in this apoptosis. COX inhibitors such as aspirin (ASP) and indomethacin (IND) prevented TAM-induced cell death and DNA fragmentation in a dose-dependent manner. On the other hand, general LOX inhibitors such as 5,8,11-eicosatrienoic acid (ETI) and nordihydroguaiaretic acid (NDGA) did not inhibit TAM-induced cell death and DNA fragmentation, although the concentrations of ETI and NDGA used in this experiment were sufficient to inhibit LOX activity.

Phase-contrast microscopic investigation showed that TAM-elicited dying cells were rounded up and detached from the bottom of the culture dish. In the presence of 2×10^{-3} M ASP or 5×10^{-5} M IND, the number of dying cells were greatly reduced. In contrast, ETI and NDGA did not show any protective effects on TAM-induced apoptosis. These results suggest that the COX pathway plays a crucial role in TAM-induced apoptosis.

TGF- β 1 induces apoptosis in K2 cells

It is widely known that TGF- β 1 is a potent growth inhibitor of hepatocytes and causes apoptosis in hepatocytes and hepatoma cells^{19,20)}. To compare the characteristics of TAM-induced apoptosis with those of the cell death induced TGF- β 1, we examined the cytotoxic effect of TGF- β 1 on K2 cells was assayed by the released lactate dehydrogenates (LDH) activity into medium. In the presence of 5 ng/ml TGF- β 1, the released LDH activity was increased in a time-dependent manner and released a plateau after 72 hr (Fig. 1A). The activity was also dose-dependently increased by the continuous treatment of 1.25-10 ng/ml TGF- β 1 (Fig. 1B). To examine whether the cell death induced by TGF- β 1 is apoptosis, we analyzed the DNA fragmentation profile in the cells treated with 5 ng/ml TGF- β 1 for various times. A substantial amount

Fig. 1 Induction of K2 cell death by TGF- β 1. K2 cells were seeded at 0.3×10^{-5} cells/well of collagen-coated 24-well plate and cultivated in Dulbecco's modified Eagle's medium containing 1% fetal calf serum for 24 hr. Then the cells were treated without or with 5 ng/ml TGF- β 1 for various times (A), and treated with various concentrations of TGF- β 1 for 72 hr (B). The released LDH activity into medium was assayed according to the method of Wroblewski and La D³⁶. Each value is the average $\pm$ SE of triplicate wells.

of DNA was cleaved into a typical DNA of oligosomal fragments within 72 hr (Fig. 2A). Staining of TGF- β 1-treated cells with Hoechst 33258 showed that in conjunction with the induction of internucleosomal DNA fragmentation, the cells expressed typical morphological features of apoptosis such as nuclear fragmentation and formation of apoptotic bodies (Fig. 2B). These results indicate that K2 cell death elicited by TGF- β 1 is a typical apoptosis.

Stimulation of [3 H]AA release by TAM and TGF- β 1

To examine whether AA cascade in K2 cells is actually triggered by TAM and TGF- β 1, we studied the effect of TAM and TGF- β 1 on the release of [3 H]AA from prelabeled K2 cells. Upon the exposure of 5×10^{-6} M TAM or 5 ng/ml TGF- β 1 to K2 cells for 2 h, a substantial amount of [3 H]AA was released into medium. The amounts of released [3 H]AA by TAM and TGF- β 1 were 2.2 and 2.0-fold higher than that of the nontreated control, respectively. The release of [3 H]AA by TAM was suppressed by the addition of 1×10^{-6} M DEX. The simultane-

Fig. 2 Induction of DNA cleavage and nuclear fragmentation by TGF- β 1. (A) K2 cells were treated with 5 ng/ml TGF- β 1 for various times and then DNA fragmentation was analyzed by 2% agarose gel electrophoresis. (B) K2 cells were cultivated without or with 5 ng/ml TGF- β 1 for 48 hr and morphological changes of the cells were observed under fluorescent microscope after staining with Hoechst 33258. Magnification, $\times 400$.

ous treatment of prelabeled K2 cells with 1×10^{-6} M each of 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), a direct activator of protein kinase C (PKC) and A23187, a Ca^{2+} ionophore, resulted in 11.4-fold increase in [^3H]AA release as compared with that of the control. It has been shown in a variety of cells that TPA synergized with A23187 in AA release through the activation of cPLA₂. K2 cell death was also induced by the simultaneous treatment with TPA and A23187.

To examine whether the stimulation of AA release by TAM is really mediated by cPLA₂, the enzyme activities in K2 cell lysates prepared after the treatment with or without 5×10^{-6} M TAM for 2 hr were assayed using 1-acyl-2- [^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine as a substrate. Although the basal level of cPLA₂ activity in K2 cells was extremely lower than those in other cell lines, the liberation of [^{14}C]AA was occurred as a dose-dependent manner and reached a plateau by the addition of 10 μl the cell lysate. When cPLA₂ activity was assayed using 5 μl of the cell lysate (corresponding to 5×10^4 cells), the enzyme activity in TAM-treated K2 cells was 28% higher than that in the vehicle control. This increased level of the enzyme activity in TAM-treated K2 cells might be enough to explain the 2.2-fold accumulation of [^3H] AA released. Thus, it is conceivable that in K2 cells TAM and TGF- β 1 stimulate AA release through the activation of cPLA₂ and AA metabolites induce K2 cell death.

PGJ₂ and Δ^{12} -PGJ₂ induce K2 cell death

The data described above suggest AA metabolites formed by the COX pathway play a crucial role in the cell death pathway of TAM and TGF- β 1. Moreover, it has been reported that PGs such as PGE₂, PGF_{2 α} and Δ^{12} -PGJ₂ induce apoptosis in murine B lymphocytes⁽³³⁾, ewe luteal cells⁽³⁴⁾ and human hepatocarcinoma SK-HEP-1 cells⁽³⁵⁾, respectively. We therefore examined the cytotoxic effects of PGE₂, PGF_{2 α} , PGJ₂ and Δ^{12} -PGJ₂ on K2 cells. Treatment with 1×10^{-4} M PGJ₂ and 1×10^{-4} M Δ^{12} -PGJ₂ for 48 h, but not PGE₂ and PGF_{2 α} , induced substantial K2 cell death

as well as the simultaneous treatment with 1×10^{-6} M each of TPA and A23187. The fraction of viable cells was time-dependently reduced and reached $23 \pm 2\%$ and $15 \pm 6\%$ after 48 hr of the treatment of 1×10^{-4} M PGJ₂ and 1×10^{-4} M Δ^{12} -PGJ₂, respectively.

Phase-contrast microscopic analysis showed that the dying cells elicited by the treatment of 1×10^{-4} M PGJ₂ for 48 hr were rounded up and detached from the culture dish. On the other hand, 1×10^{-4} M PGE₂ or PGF_{2 α} did not show any cytotoxic effects. Electron microscopic study indicated that significant chromatin condensation, nuclear fragmentation and apoptotic bodies were caused in K2 cells treated with 1×10^{-4} M PGJ₂ for 48 hr, suggesting that PGJ₂ could induce apoptotic cell death. Similar morphological changes were observed in K2 cells treated with 1×10^{-4} M Δ^{12} -PGJ₂.

Conclusion

Here, we show that an antiestrogen, TAM, induces apoptosis in AFB₁-induced rat hepatoma K2 cells existing in G₁ and/or S phase, and this apoptosis can be efficiently prevented by GCs, but not other steroid compounds including E₂, possibly due to the inhibition of [³H]AA release. Furthermore, the experiments using COX inhibitors (ASP and IND) and LOX inhibitors (ETI and NDGA) indicate that the COX pathway (in particular, the PGJ₂ and Δ^{12} -PGJ₂ pathways) of AA metabolism may at least in part, play a role in the apoptotic cell death triggered by TAM as well as TGF- β 1, an inducer of apoptosis in hepatocytes and hepatoma cells. In addition to the reported functions of endocrine factors including effects of AFB₁ metabolism and uptake of AFB₁^{13,14}, our present data offer the new hypothesis that GCs could act as tumor promoters in AFB₁ hepatocarcinogenesis.

It is well established that endocrine factors play an important role in carcinogenesis. Goodall and Butler¹³ reported that hypophysectomy in rat prevents liver carcinogenesis developing from the administration of AFB₁ due to the reduction of metabolic activation of AFB₁. Neal and Judah¹⁴ also reported that hypophysectomy could protect against the cytotoxic and carcinogenic actions of AFB₁ by removing the steroid uptake mechanism by which AFB₁ enters the subcellular structures. In addition to these facts, our results suggest the possibility that endocrine factors, especially GCs, affect AFB₁-induced hepatocarcinogenesis through the prevention of apoptosis induced by AA metabolites formed *in vivo*.

In conclusion, these facts and our present data, it is possible that GCs promote AFB₁ hepatocarcinogenesis through the prevention of apoptosis induced by various cellular defense systems including TGF- β 1 and perhaps TNF, whose actions are mediated at least in part by AA metabolites.

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グルココルチコイドホルモンはアフラトキシン B₁の肝癌誘発過程において発癌プロモーターとして関与している可能性がある

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