Analysis of cell death induced by sodium 5,6-benzylidene-ascorbate in rat hepatoma Kagura-2 cells

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Summary

Aflatoxin B1 (AFB1)-induced hepatocellular carcinoma Kagura-2 cells overexpress c-myc oncogene, which has been implicated in both cell proliferation and apoptosis. To gain an insight into the molecular mechanism of AFB1 hepatocarcinogenesis, Kagura-2 cell death induced by an anticancer agent, sodium 5,6-benzylideneascorbate (SBA), was studied. The dying cells showed typical characteristics of apoptosis such as nuclear fragmentation, chromosomal condensation and DNA fragmentation by internucleosomal cleavage. The apoptotic death was inhibited by the addition of cycloheximide (CHX), suggesting the requirement for new protein synthesis. However, the induction of Ca2+/Mg2+-dependent endonuclease activities and the alteration of c-myc gene expression in SBA-induced apoptosis were hardly detected. In addition, SBA-induced apoptosis was markedly suppressed by dexamethasone (DEX), insulin and P3 fraction, which was separated from the conditioned medium of Kagura-2 cells (K2CM) by Sephadex G-200 column chromatography and could stimulate Kagura-2 cell growth. P3 fraction also inhibited DNA fragmentation of Kagura-2 cells induced by serum deprivation. These results suggest that SBA induces apoptosis through the interference with the function of growth/survival factors acting in an autocrine and/or a paracrine mechanism or their signal transduction pathways. It is also possible that growth/survival factors play a critical role in the multistep hepatocarcinogenesis of AFB1 together with the deregulated expression of c-myc oncogene.

Key words: sodium 5,6-benzylideneascorbate, 5,6-ベンジリデンアスコルビン酸ナトリウム；cell death, 細胞死；apoptosis, アポトーシス；aflatoxin B1, growth/survival factor, 成長/生存因子

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Apoptosis, a form of cell death in which the cell actively participates in its demise, is characterized by chromatin condensation, DNA fragmentation and cytoplasmic blebbing. The process of apoptosis is frequently dependent on RNA and protein synthesis in the dying cells. Apoptosis is essential in many aspects of normal development and is required for maintaining homeostasis. Failure to regulate apoptosis positively is associated with cancer and autoimmune disease, and failure to regulate apoptosis negatively is associated with degenerative diseases. Many reports for apoptosis in diverse systems suggest the following mechanisms: (1) Apoptosis may arise from incompatible or conflicting growth signals; (2) multiple pathways exist for induction of apoptosis; and (3) apoptosis can be a multistep process.

In mammalian cells, gene products that either activate or suppress apoptosis have been identified. The product of p53 tumor suppressor gene has been shown to be responsible for directing apoptosis in various cells, and the results suggest that apoptosis is a cellular defence against cancer. Deregulated c-myc oncogene expression is associated with stimulation of both cell proliferation and apoptosis. Induction of apoptosis is thereby associated with the deregulation of cell growth control, that would occur in progressing neoplasia. p53 tumor suppressor gene may be responsible for responding to abnormal growth situations by initiating apoptosis. Moreover, the specific factor required for factor-dependent survival of cells expressing deregulated c-myc gene is determined in some cell lines [i.e., insulin-like growth factor-I (IGF-I) and platelet-derived growth factor (PDGF) in Rat-1 fibroblasts, interleukin-3 (IL-3) in IL-3 responsive hematopoietic cells]. Thus, growth factros and cytokines play an important regulatory role as a survival factor.

To clarify the detailed mechanism of aflatoxin B1 (AFB1) hepatocarcinogenesis, we have established the hepatoma cell line designated as Kagura-2 from AFB1-induced rat hepatocellular carcinoma. The cells overexpress c-myc oncogene and develop solid tumor when transplanted into the flank of nude mice. The colony formation of Kagura-2 cells in semisolid medium is significantly enhanced by dexamethasone (DEX), one of the synthetic glucocorticoid hormones, and the cells show a DEX-dependent growth in serum-free medium. The cells secrete a 17 kDa neuronal differentiation factor and at least three kinds of growth factors including a transforming growth factor-β (TGF-β)-like factor, a fibroblast growth factor (FGF)-like factor and an unknown growth inhibitory factor. The inhibitory activity existing in the conditioned medium of Kagura-2 cells (K2CM) in the presence of DEX tends to be lower than that in the absence of DEX. Therefore, it seems likely that DEX-dependent growth of Kagura-2 cells is regulated through these stimulatory and inhibitory factors in an autocrine and/or paracrine mechanism.

A benzaldehyde derivative, sodium 5,6-benzylideneascorbate (SBA) has been used for the chemotherapy of inoperable human cancers of ovary, stomach, pancreas, uterus, bile duct and lung without serious side effects. Recently, it has been demonstrated that SBA degenerates rat hepatocellular carcinomas induced by 3'-methyl-4-dimethylaminoazobenzene and induces apoptosis in mouse L929 cells.

In this paper, to clarify the detailed mechanism of AFB1 hepatocarcinogenesis, we investigated the cell death of Kagura-2 cells primed by SBA. The results indicate that the form of cell death is apoptosis but not necrosis, and the apoptosis is efficiently prevented by growth/survival.
factors existing in K2CM. The possible roles of growth/survival factors in AFB\textsubscript{1} hepatocarcinogenesis were discussed.

**Materials and Methods**

**Materials**  SBA as shown in Fig. 1 was supplied by ChemiScience and dissolved in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free phosphate buffered saline [PBS (\textsuperscript{−})] for experiments. DEX, insulin and Hoechst 33258 were purchased from Sigma. Recombinant basic FGF (bFGF) was obtained from Oncogene Science.

**Cell culture**  Kagura-2 cells were cultivated in collagen-coated dishes with Dulbecco's modification of Eagle's medium supplemented with 5\% fetal calf serum (FCS). The cells were maintained at 37\°C in a humidified atmosphere of 5\% CO\textsubscript{2} in air. Cell viability was determined by exclusion of 0.2\% trypan blue. K2CM was fractionated by Sephadex G-200 column chromatography as described previously\textsuperscript{11}.

**Colony formation assay**  Kagura-2 cells were seeded at 1.0 × 10\textsuperscript{5} cells/35-mm dish and cultivated for 24 hr and then treated with 0.5 mg/ml of SBA for various times. The cells were replated 2.0 × 10\textsuperscript{4} cells/35-mm dish and cultivated for 4 days without SBA. Colonies made up more than 10 cells were counted.

**Analysis of DNA fragmentation**  Kagura-2 cells were seeded at 1.0 × 10\textsuperscript{5} cells/35-mm dish, cultivated for 24 hr and then treated with various concentrations of SBA for 48 hr. Both detached and attached cells were simultaneously harvested and centrifuged at 400 \times g for 5 min. The resulting pellets were rinsed once with PBS(\textsuperscript{−}), suspended in 100 \mu l PBS(\textsuperscript{−}), and then lysed by the addition of the same volume of the 2\times lysis buffer [100 mM Tris-HCl (pH 7.8)-20 mM EDTA-1\% (W/V) sodium N-lauroyl sarcosinate]. The lysates were incubated sequentially with 200 \mu g/ml RNase A for 15 min at 50\°C and 200 \mu g/ml proteinase K for 6-12 hr at 50\°C. Electrophoresis of the fragmented DNA (10 \mu g) was carried out in 2\% agarose gel in TBE buffer [89 mM Tris-borate (pH 8.0)-2 mM EDTA] at 100 V for 2 hr. The gels were stained with 0.5 \mu g/ml of ethidium bromide for 30 min and destained with distilled water for 2 hr. The photographs of DNA fragments were taken under UV illuminator.

![Chemical structure of SBA.](image)
Staining of cellular nuclei with Hoechst 33258  Kagura-2 cells were cultivated on collagen-coated cover glasses, which were pretreated with 0.1% acetic acid for 30 min at 100°C, and treated with 0.5 mg/ml of SBA for 48 hr. Then the cells were rinsed with PBS(−) and fixed with the fixation buffer [0.1 M sodium cacodylate-HCl (pH 7.4)-2% paraformaldehyde-1% glutaraldehyde] for 30 min at room temperature. After the removal of the fixation buffer, the cells were stained with 0.1 μg/ml of Hoechst 33258 for 30 min and then overlayed with the sealing buffer [50% (V/V) glycerol-25 mM citric acid-50 mM sodium phosphate (pH 4.9)]. The stained cells were examined under a BH-2 fluorescent microscope (Olympus).

Electron microscopic analysis  Cells fixed in the fixation buffer [0.1 M sodium cacodylate-HCl (pH 7.4)-1.5% (V/V) formaldehyde-1% glutaraldehyde] more than 6 hr at 4°C were rinsed in 0.1 M cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 hr at 4°C, and then dehydrated in sequential ethanol series. The cells were infiltrated, embedded in Epon 812, and sectioned. The sections were double stained with uranyl acetate and lead acetate, and examined under a 1200 EX electron microscope (JEOL).

Electrophoretic analysis of nuclease  Nucleases were extracted from the nuclei isolated from SBA-treated cells. The cells were swelled in 10 mM potassium phosphate (pH 8.0)-10 mM NaCl-1.5 mM MgCl₂-0.5 mM dithiothreitol-30 μg/ml phenylmethyl sulfonylfluorid (PMSF) buffer and homogenized with a teflon homogenizer. The homogenates were overlayed on 1 M sucrose and centrifuged at 8,000×g for 30 min. The nuclear pellets were mixed with 0.6 M NaCl-10 mM Tris-HCl (pH 7.4)-15 μg/ml PMSF buffer on a rotator for 1 hr to prepare nuclear extracts and centrifuged at 15,000×g for 15 min. The resulting supernatants were used for the analysis of endonucleases according to the method reported by Compton and Cidlowski. The nuclear extracts were electrophoresed on 12.5% SDS-polyacrylamide gel containing 10 μg/ml of calf thymus DNA. Following electrophoresis, SDS was removed from the gel by soaking with 40 mM Tris-HCl (pH 7.6)-2 mM MgCl₂-0.02% (W/V) NaN₃ buffer overnight. Nucleases were activated by the addition of 2 mM CaCl₂ to the above solution and incubated for 45 min at room temperature. Then the gel was stained with 1 μg/ml of ethidium bromide. Nuclease activities were visualized as a darkened bands under UV illuminator. Micrococcal nuclease was used as a positive control for these experiments.

Results

Effect of SBA on cell viability and proliferation  In this experiment, we first examined the cytotoxic effect of SBA on Kagura-2 cells assessed by the trypan blue exclusion method. The ratio of viable cells was dose-dependently depressed by the continuous treatment of SBA for 48 hr as shown in Fig. 2-A. The maximal depression observed at 1 mg/ml of SBA was determined to be 48% of the control, but no further cytotoxic effect was observed at 2 mg/ml SBA. Therefore, 0.5 mg/ml of SBA was used for main subsequent studies. As shown in Fig. 2-B, the time course experiment indicated that the rate of cell death primed by SBA was markedly accelerated after 4 days of treatment and >90% of the treated cells were killed after 12 days. These results suggest that the early effect of SBA is not simply associated with loss of membrane integrity.
Fig. 2 Cytotoxicity of SBA.

(A) Kagura-2 cells were seeded at $1.0 \times 10^5$ cells/35-mm dish and cultivated for 24 hr. Then the cells were treated with various concentrations of SBA for 48 hr. Cell viability was determined by the trypan blue exclusion method. Each value is the average ± SE of triplicate culture dishes. *, $P<0.045$; **, $P<0.001$ compared with the control.

(B) Kagura-2 cells were cultivated with or without 0.5 mg/ml of SBA for various times as indicated in the figure. Each value is the average ± SE of triplicate culture dishes. *, $P<0.005$; **, $P<0.001$ compared with day 0.

Fig. 3 Inhibitory effect of SBA on colony formation.
Kagura-2 cells were treated with 0.5 mg/ml of SBA for various times. The cells were replated at $2 \times 10^5$ cells/35-mm dish and cultivated for 4 days. Colonies made up more than 10 cells were counted. Each value is the average of triplicate culture dishes. SE ± 1 for all values. *, $P<0.01$, **, $P<0.001$ compared with time 0.
Fig. 4 Induction of DNA fragmentation by SBA. Kagura-2 cells were treated with various concentrations of SBA for 48 hr. DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane 1 indicates the size marker. Lanes 2 to 7 indicate 0, 0.125, 0.5, 1.0 and 2.0 mg/ml of SBA, respectively.

To examine the early effect of SBA on Kagura-2 cells, the colony formation assay was carried out. As shown in Fig. 3, the colony formation ability of Kagura-2 cells was reduced depending upon the exposure time to SBA. In contrast to the results of the time course experiment of cytotoxicity, only after 3 hr of the exposure to SBA, the colony formation ability was inhibited >20% as compared with the control and >80% of inhibition was observed after 48 hr of the treatment. The results indicate that SBA irreversibly interferes with the growth regulatory system of Kagura-2 cells shortly after the treatment and ultimately induces the cell death assessed by the trypan blue exclusion method.

Evidence of apoptotic cell death To examine whether the cell death induced by SBA is apoptosis, we first analyzed the DNA fragmentation profile in the cells treated with various concentrations of SBA for 48 hr. As shown in Fig. 4, a substantial amount of DNA was cleaved into a typical DNA of oligonucleosomal fragments. When the cells were treated with 0.5 mg/ml of SBA, DNA fragmentation was detectable within 12 hr (data not shown).

The morphological changes in SBA-treated cells were also investigated. Phase-contrast microscopic investigation revealed that the dying cells were shrunken and rounded up, giving the appearance of membrane blebbing into cellular fragments (Fig. 5-B). Staining of SBA-treated cells with Hoechst 33258 showed that in conjunction with the induction of internucleosomal DNA fragmentation, the cells expressed extensive morphological features of apoptosis such as nuclear fragmentation and formation of apoptotic bodies (Fig. 5-D). Electron microscopic analysis indicated that significant chromatin condensation and nuclear fragmentation were evoked by SBA (Fig. 5-F). Based on the observations of DNA fragmentation, chromatin condensation and morphological changes in SBA-treated Kagura-2 cells, it was demonstrated that the cell death of Kagura-2 cells induced by SBA is a typical apoptosis.

Inhibition of SBA-induced apoptosis by cycloheximide (CHX) Apoptosis often requires new
protein synthesis, although there is evidence to support the notion that is no universal mechanism for apoptosis\(^9\). Therefore, the effect of CHX on DNA fragmentation induced by SBA was examined. As shown in Fig. 6, the apoptotic degradation of DNA in SBA-treated Kagura-2 cells was efficiently blocked by the addition of 0.1 \(\mu\text{g/ml}\) CHX during before 1 hr and after 3 hr of the exposure to SBA. However, when CHX was added to the cell culture after 6 hr of the SBA treatment, its inhibitory activity completely disappeared. The results suggest that SBA requires new protein synthesis to induce apoptosis and commits to apoptotic cell death within 6 hr.
Fig. 6 Effect of CHX on SBA-induced apoptosis.
CHX (100 ng/ml) was added at various times as indicated before or after the addition of 0.5 mg/ml of SBA. After 48 hr of the SBA treatment, DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane 1, size marker; lane 2, control; lane 3, CHX only; lane 4, CHX added 1 hr before; lanes 5 to 12, CHX added 0, 0.5, 1, 3, 6, 12, 24, and 36 hr after the addition of SBA, respectively.

Fig. 7 Analysis of endonuclease activities in SBA-treated cells.
Kagura-2 cells were treated with 0.5 mg/ml SBA for various times and then nuclei were isolated. Nucleases were extracted from the isolated nuclei with 0.6 M NaCl. The extracts were electrophoresed on 12.5% SDS-polyacrylamide gel containing 10 μg/ml of calf thymus DNA. Nuclease activities were assayed in the presence of 2 mM each of Mg²⁺ and Ca²⁺, and visualized under UV illuminator (A). Coomassie brilliant blue-stained gel is also shown to allow comparison of total amount of protein employed (B). Lane 1, size marker; lanes 2 and 3, 1 and 2 μg/ml of micrococal nuclease, respectively; lane 4, control; lanes 5 to 7, nuclear extracts from the cells treated with SBA for 12, 24 and 48 hr, respectively.

Effect of SBA on endonuclease activity and c-myc gene expression In thymocytes it has been demonstrated that endonuclease activities are induced in the early stage of apoptosis primed by DEX⁹. In addition, SBA-induced apoptosis was significantly inhibited by CHX as described above. We analyzed endonucleases in SBA-treated Kagura-2 cells. Nuclear proteins were extracted from the cells treated with 0.5 mg/ml of SBA for 12, 24 and 48 hr and then were analyzed on 12.5% SDS-polyacrylamide gels for nuclease activities. As shown in Fig. 7, any changes of endonuclease activities were not observed.
Fig. 8 Effect of SBA on c-myc gene expression. Kagura-2 cells were treated with 0.5 mg/ml of SBA for various times as indicated. Total RNAs were extracted and examined by Northern hybridization as previously reported (A). Picture of ethidium bromide stained gel is included to allow comparison of total amount of RNA employed (B).

Since deregulated expression of c-myc oncogene can induce apoptosis in Rat-1 cells and hematopoietic cells in the absence of serum or growth factors, we analyzed the effect of SBA on the expression of c-myc gene in Kagura-2 cells by Northern hybridization. As shown in Fig. 8, significant change of c-myc gene expression was not detected in SBA-treated cells.

**Inhibition of SBA-induced apoptosis by hormones** It has been reported that c-myc-induced apoptosis in Rat-1 cells is inhibited by IGF-I and PDGF. We previously found that DEX and insulin, which play an important role in the regulation of glyconeogenesis in liver tissue, act as a growth factor for Kagura-2 cells. We therefore examined the effect of insulin and DEX on SBA-induced apoptosis. As shown in Fig. 9, the apoptotic cell death induced by SBA was significantly suppressed in the presence of 2.5×10^{-6} M insulin and/or 1.0×10^{-7} M DEX (Fig. 9-A). Apoptotic DNA degradation by SBA was also dose-dependently inhibited by insulin and DEX, as shown in Fig. 9-B and 9-C, respectively.

**Protection of apoptosis by K2CM** When K2CM was fractionated by Sephadex G-200 column chromatography, three main peaks (P1, P2 and P3) were detected by the assay of DNA synthesis in Balb/3T3 cells as described previously. P3 fraction had a strong stimulatory effect on DNA synthesis in Balb/3T3 cells, while P1 fraction showed an inhibitory effect. We recently found that all of these peak fractions could stimulate the growth of Kagura-2 cells as shown in Fig. 10-A. The cell death of Kagura-2 was induced within 4 days after the serum starvation, possibly owing to its deregulated expression of c-myc gene (data not shown). Furthermore, in the dying
Fig. 9 Inhibitory effect of insulin and DEX on SBA-induced apoptosis. Kagura-2 cells were treated with 0.5 mg/ml of SBA together with $2.5 \times 10^{-6}$ M insulin and/or $1.0 \times 10^{-7}$ M dex for 48 hr. (A) Cell viability was determined by the trypan blue exclusion method. Each value is the average±SE of triplicate culture dishes. *, P<0.05 compared with the SBA-treated control. (B) Kagura-2 cells were treated with 0.5 mg/ml SBA in the presence of various concentrations of insulin for 48 hr and then DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane 1, size marker; lane 2, control; lane 3, SBA only; lanes 4 to 8, treated with SBA in the presence of $1.0 \times 10^{-7}$, $1.0 \times 10^{-6}$, $2.5 \times 10^{-6}$, $1.0 \times 10^{-5}$ and $2.5 \times 10^{-5}$ M insulin, respectively. (C) Kagura-2 cells were treated with 0.5 mg/ml of SBA in the presence of various concentrations of DEX for 48 hr and then DNA fragmentation was analyzed as described above. Lane 1, size marker; lane 2, control; lanes 3 to 5, treated with SBA in the presence of $1.0 \times 10^{-8}$, $1.0 \times 10^{-7}$ and $1.0 \times 10^{-6}$ M, respectively; lane 6, SBA only.
Fig. 10 Effect of K2CM on cell growth.

(A) K2CM was fractionated by Sephadex G-200 column chromatography as described previously\textsuperscript{11}. Each fraction was assayed for the cell growth of Kagura-2 cells and DNA synthesis in Balb/3T3 cells. Each value is the average of duplicate culture dishes.

(B) Kagura-2 cells were cultivated in serum-free medium supplemented with P1, P2 or P3 fraction for 6 days and then DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane 1, size marker; lane 2, serum-starved control; lanes 3 to 5, addition of P1, P2 and P3 fractions, respectively.

cells DNA fragmentation was detected, showing that the cell death induced by serum starvation is apoptosis. As shown in Fig. 10-B, P1, P2 and P3 fractions efficiently inhibited DNA fragmentation induced by serum starvation. This result suggests the possibility that these three fractions act as a survival factor against apoptosis.

These results led us to examine whether the three fractions inhibit apoptosis induced by SBA. As shown in Fig. 11-A, P3 fraction significantly inhibited SBA-induced cell death, while P1 and P2 fractions did not show an inhibitory effect. P3 fraction also suppressed DNA fragmentation primed by SBA. The data suggest that SBA may induce apoptosis in Kagura-2 cells through the interference with the function of growth/survival factors or their signal transduction.
Fig. 11 Effect of K2CM fractions on SBA-induced apoptosis. Kagura-2 cells were treated with 0.5 mg/ml of SBA in the presence of 25 μg/ml each of P1, P2 or P3 fraction for 48 hr. (A) Cell viability was determined by trypan blue exclusion method. Each value is the average ± SE of triplicate culture dishes. *, P<0.03; **, P<0.001 compared with the control. (B) DNA fragmentation was analyzed by 2% agarose gel electrophoresis. Lane 1, size marker; lane 2, control; lane 3, SBA only; lanes 4 to 6, treated with SBA in the presence of P1, P2 and P3 fractions, respectively.

Fig. 12 Effect of bFGF and insulin on DNA synthesis in Kagura-2 cells. Kagura-2 cells were treated with 50 ng/ml of bFGF or 2.5×10⁻⁶ M insulin for 48 hr and then the incorporation of ³H-thymidine into acid-insoluble fraction was determined as described previously. Each value is the average ± SE of triplicate culture dishes. *, P<0.05; **, P<0.001 compared with the control.

P3 fraction contains a FGF-like factor, which strongly binds to heparin-Sepharose column and is eluted with 2 M NaCl. Accordingly, we examined the effect of recombinant bFGF on pathways.
the DNA synthesis in Kagura-2 cells. As shown in Fig. 12, bFGF stimulated DNA synthesis as well as insulin. However bFGF exhibited no effect on SBA-induced apoptosis in Kagura-2 cells (data not shown). The data support the idea that the function and signal transduction pathway of growth factors are distinct from those of survival factors as reported by Yao and Cooper.

Discussion

In this paper, we clearly demonstrated that the cell death induced by SBA in Kagura-2 cells expressing the c-myc gene is a typical apoptosis on the basis of nuclear fragmentation, chromatin condensation, DNA fragmentation and cellular membrane blebbing, although the induction of nuclear endonuclease activities was not observed. Moreover, SBA-induced apoptosis was efficiently suppressed by the addition of CHX, indicating the requirement for new protein synthesis.

It is well known that c-myc is an early response gene which is associated with cell proliferation but is oncogenic when its expression is deregulated. The expression of c-myc gene is normally induced in proliferating cells following mitogenic stimulation and is down-regulated after serum or growth factor deprivation, whereupon cells arrest in G1 phase. Deregulated c-myc expression, which prevents down-regulation of c-myc level after serum or growth factor deprivation, induces apoptosis through the wild type of tumor suppressor gene p53, but not growth arrest. The inability of cells with deregulated c-myc to arrest upon serum or factor deprivation suggests that apoptosis is caused by incompatible growth signals resulting from the signal to proliferate from c-myc and the signal to arrest from serum or growth factor deprivation. These conflicting growth situations may be irreconcilable, causing cell death. Therefore, the strict regulation of c-myc gene expression may be required during normal cell growth, because c-myc induces both proliferation and apoptosis. The specific factor required for the survival of cells expressing deregulated c-myc is clarified in Rat-1 cells. This may represent a safety mechanism for checking inappropriate cell proliferation involving c-myc.

In this regard, we found that SBA-induced apoptosis in Kagura-2 cells having wild type p53 gene, was significantly inhibited by a synthetic glucocorticoid, DEX, insulin, and P3 fraction of K2CM which can stimulate the growth of Kagura-2 cells and inhibit apoptosis induced by serum deprivation. Furthermore, tamoxifen-induced apoptosis was suppressed by the addition of antisense c-myc oligonucleotides (Myobudani et al., unpublished observation). Taking into many other reports described above and our results, it seems likely that c-myc plays a critical role in SBA-induced apoptosis in Kagura-2 cells. It is also possible to postulate that SBA induces apoptosis through the interference with the function of growth/survival factors acting in an autocrine and/or a paracrine manner or their signal transduction pathways as one of the modes of action of SBA.

P3 fraction prepared from K2CM by Sephadex G-200 column chromatography contains a FGF-like factor and a 17 kDa neuronal differentiation factor, which strongly bind to heparin. Therefore, we examined the effect of recombinant bFGF on DNA synthesis and SBA-induced apoptosis in Kagura-2 cells. bFGF significantly enhanced DNA synthesis but did not inhibit apoptosis induced by SBA, although P3 fraction acting as a survival factor for SBA-induced
apoptosis. These data suggest that at least the FGF-like factor in P3 fraction is not bFGF, and also indicate the possibility that the survival ability of growth factors to inhibit apoptosis is not simply dependent on their competence to promote cell growth.

The neurotrophic factors including nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3), NT-4 and NT-5 utilize the trk protooncogene family of receptor tyrosine kinase and act as a neuronal survival factor\textsuperscript{19}. When trk A gene encoding NGF receptor was expressed in NIH/3T3 fibroblasts, NGF is a powerful mitogen that can induce resting NIH/3T3 cells to enter S phase, grow in semisolid medium, and become morphologically transformed\textsuperscript{21}. The similar effect was reported for BDNF\textsuperscript{22}. Recently, we detected the expression of trk B gene encoding the receptor for BDNF and NT-5 in Kagura-2 cells\textsuperscript{20}. The level of trk B mRNA in Kagura-2 cells was higher than that of adult rat brain\textsuperscript{20}. In addition, the 17 kDa neuronal differentiation factor is thought to be a factor related to NGF family\textsuperscript{10}. Although the survival activity of the 17 kDa neuronal differentiation factor for Kagura-2 cells has not been examined yet due to the difficulty of its purification, it seems likely that the real survival factor of P3 fraction against apoptosis induced by SBA is the 17 kDa factor.

Verification of various cooperative genetic lesion in multiple steps of carcinogenesis is a major issue in cancer research. Cooperating oncogenes are categorized as those which complement each other in signal transduction pathways of many growth factors\textsuperscript{20}. This means that the growth advantage acquired the synergic action of multiple oncogenes is the results of a balanced mitogenic signal which constitutively stimulates both cell cycle progression and cell survival. Although only overexpression of c-myc gene without serum causes apoptosis, c-myc gene seems to be very important in oncogene synergy\textsuperscript{24}. It has been reported that c-myc synergizes with ras, raf and anti-apoptotic bcl-2 oncogenes in cell transformation\textsuperscript{25–27}. Moreover, by its interaction with Bcl-2 protein, Raf-1 kinase has been reported to prevent myeloid cell apoptosis induced by the withdrawal of IL-3\textsuperscript{28}. Taking into these facts and our present data, it seems likely that the survival signal of P3 fraction to inhibit SBA-induced apoptosis is transferred to anti-apoptotic bcl-2 family of genes through ras and raf oncogenes involving in signal transduction pathways.

Elucidating the possible role of the survival factors in P3 fraction to inhibit SBA-induced apoptosis in Kagura-2 cells should provide further information for understanding the molecular mechanism of AFB\textsubscript{1} hepatocarcinogenesis.

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References