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Apoptosis in Human Hepatoma Cell Line Induced by 4,5-Didehydro geranylgeranoic Acid (Acyclic Retinoid) via Down-Regulation of Transforming Growth Factor-α

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Synthetic 4, 5-didehydro GGA (geranylgeranoic acid), a potent ligand both for cellular retinoic acid-binding protein and for nuclear retinoid receptors, induced apoptosis in human hepatoma-derived cell line HuH-7 but not in primary hepatocytes, although all-*trans* or 9-*cis* retinoic acid did not induce any growth inhibition. Either exogenous transforming growth factor-α (TGFα) or epidermal growth factor (EGF) prevented the cells from apoptosis in the presence of 4, 5-didehydro GGA, but hepatocyte growth factor, insulin-like growth factor-II, insulin or triiodothyronine was essentially inactive. 4, 5-Didehydro GGA down-regulated the cellular levels of TGFα mRNA as early as 30 min after the treatment. Either anti-TGFα or anti-EGF receptor monoclonal antibody induced apoptosis in HuH-7 cells without using the acid. Taken together, the present study strongly suggests that 4, 5-didehydro GGA induced apoptosis in HuH-7 cells through the destruction of autocrine loop consisting of TGFα and EGF receptor, due to the down-regulation of TGFα gene expression.

Apoptosis has been now expected to be one of the most effective mechanisms which prevent carcinogenesis (1). Many agents have so far been reported to induce apoptosis, such as, ceramide (2), ether phospholipid (3), tumor necrosis factor-α (TNFα) (4), transforming growth factor-β (TGFβ) (5), and anti-FAS antibody (6). Many anti-cancer drugs may also exert their effects through the enhanced induction of apoptosis (7). Recently we have found that a synthetic 4, 5-didehydro GGA, or previously called as 'acyclic retinoid' (8), was able to induce *apoptosis in human* hepatoma-derived cell line, HuH-7, proved by the chromatin condensation and a characteristic step-wise fragmentation of genomic DNA (8). And 4, 5-didehydro GGA efficiently prevented spontaneous hepatocarcinogenesis in C3H/HeNCrj male mice with its few doses at around 11 months after birth (9). There have been also accumulating evidence that cooperation of c-*myc* and transforming growth factor-α (TGFα) contributes to the selective expansion of hepatic preneoplastic populations, probably due to abrogation of apoptotic cell death in preneoplastic cells (10). In this communication, we demonstrate that down regulation of TGFα, an autocrine promotor for liver carcinogenesis, was a crucial step in the mechanism underlying apoptosis induced by 4, 5-didehydro GGA in HuH-7 cells.

MATERIALS AND METHODS

Materials. All-*trans* retinoic acid (RA) was purchased from Sigma Chemical Co. (St. Louis, MO). 4, 5-didehydro GGA was supplied from Eisai Co, Tokyo. These retinoids were dissolved in a 99.5% ethanol (5 vol)-dimethyl sulfoxide (1 vol) mixture at 10 mM concentration. Triiodothyronine ($T_{,3}$), epidermal growth factor (EGF), insulin was purchased from Sigma Chemical Co. TGFα was purchased from GIBCO BRL (Bethesda, MD, USA), hepatocyte growth factor (HGF) was purchased from Becton Dickson (Bedford, MA, USA), insulin-like growth factor-II (IGF-II) was purchased from Wakunaga Pharma Co. (Hiroshima, Japan). PCR primers for human TGFα human G3PDH and human β-actin were purchased from Clontech Lab. (Palo Alto, CA, USA). Monoclonal antibodies against TGFα or EGF-receptor were purchased from Oncogene Science (Cambridge, MA, USA).

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Preparation of mouse primary cultured hepatocytes. Eight-week-old male Balb/c mice were obtained from Chubu Kagaku Shizai Co. (Nagoya, Japan). Hepatocytes were isolated from mouse liver digested with collagenase perfusion.

Treatment of cells with retinoids. Human hepatoma-derived cell line, HuH-7 cells or mouse primary cultured hepatocytes were seeded at 1×10^5 cells/ml per well with α-MEM containing 10% FCS in Falcon 12-well plastic plates. The medium was removed after 24 hr and cells were washed with Hank's solutions three times and FCS-free α-MEM was added. Cells were cultured for 48 hr, and then the medium was replaced by FCS-free α-MEM containing 10 μM retinoids. In the experiment with hepatotrophic factors, each factor at a final concentration of 100 ng/ml was added 6 hrs prior to the treatment of 10 μM 4, 5-didehydro GGA. Viable cells were counted by the trypan-blue dye exclusion method at the indicated time points.

Treatment of cells with anti-TGFα or anti-EGF receptor monoclonal antibody. After the pretreatment of HuH-7 cells with FCS-free medium as mentioned above, the conditioned medium was replaced by FCS-free α-MEM containing anti-TGFα or anti-EGF receptor monoclonal antibody (100 μg/ml). Cells were counted at each day by the trypan blue dye exclusion method.

RT–PCR. Total RNA was isolated by acid guanidinium thiocyanate phenol chloroform extraction from non-treated control cells, 4, 5-didehydro GGA or all-*trans* RA treated cells. One μg RNA from each treated cell was preincubated with 1 μl (100 pmol/μl) random primer (Boeringer Mannheim, Mannheim, Germany), 1 μl 20 mM deoxynucleoside-triphosphate (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP), 4 μl reverse transcriptase buffer consisting of 250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$, and 12 μl H_2O for 2 min at 70° C. Reverse transcription was performed at 37° C for 40 min with 200 units (1 μl) Molony murine leukemia virus reverse transcriptase (Boeringer Mannheim). After transcription, cDNA was amplified with 1 μl of 20 mM deoxynucleoside-triphosphate, 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$), 1 μl 25 pM oligonucleotide primers and 66.5 μl of H_2O under standard condition supplied from Clontech. This amplification was repeated 40 times. Detection of amplification products was done by ethidium bromide staining in 1% agarose gel after electrophoresis.

Northern blot analysis. Total RNA was separated by 1.2% agarose, 1% formaldehyde gel electrophoresis, transferred to Hybond-N (Amersham, Buckinghamshire, UK), and hybridized to *c-mcy* exon-2 DNA probe labeled with [α-^{32}P] dCTP using random primers (Amersham).

RESULTS AND DISCUSSION

4, 5-didehydro GGA (or previously called ''acyclic retinoid'') has been reported as a potent inducer for apoptosis in HuH-7 cells (8). In this report, we tested the ability of 4, 5-didehydro GGA to induce apoptosis in normal hepatocytes. As shown in Fig. 1, mouse primary cultured hepatocytes were totally insensitive to the acid, whereas at the same condition HuH-7 cells died by apoptosis. Primary hepatocytes were not proliferating but HuH-7 cells were mitogenic in FCS-free medium. Therefore, we speculate that HuH-7 cells produce autocrine growth factor(s) and certain growth factor must be involved in the cellular process of anti-apoptotic signal transduction.

Prior to exploring the putative autocrine growth factor(s), we first tried six hepatotrophic factors

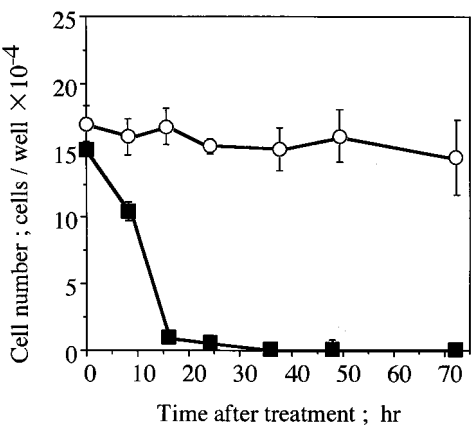

FIG. 1. Time course of cell survival after 4, 5-didehydro GGA treatment in HuH-7 cells or primary cultured hepatocytes. The cells (■; HuH-7, ○; mouse primary cultured hepatocytes) were treated with 10 μM 4, 5-didehydro GGA. Viable cells were counted by the trypan blue dye exclusion method at the indicated time points. Each point represents the mean ± S.E. (n = 3).

such as insulin, T_3, TGFα, EGF, HGF, and IGF-II (11), to assess whether or not they could prevent the cells from apoptosis in the presence of 4, 5-didehydro GGA. As shown in Fig. 2, only TGFα or EGF inhibited 4, 5-didehydro GGA-mediated apoptosis, whereas the other hepatotrophic factors used here failed. It was quite reasonable to speculate that HuH-7 cells may express and secrete TGFα and the autocrine TGFα might be ''anti-apoptotic'' or ''survival'' signal for the cells in serum-free medium. In this context, we speculate that 4, 5-didehydro GGA may cause down-regulation of TGFα expression, which might be sufficient for HuH-7 cells to die by apoptotic mechanism.

We investigated the effects of 4, 5-didehydro GGA on the expression of TGFα mRNA by RT-PCR, because Northern blot hybridization failed to detect TGFα mRNA. As a result of 40 cycled-PCR, we could clearly show that HuH-7 cells expressed the mRNA for TGFα and in control or all-*trans* RA-treated cells, its cellular level remained constant during the experiment as well as two other typical house-keeping genes such as G3PDH and β-actin. However, Fig. 3 clearly indicated that 4, 5-didehydro GGA remarkably reduced the intracellular level of TGFα mRNA. The expression of TGFα was rapidly reduced and this down-regulation lasted until 6 hr as long as we observed. Consequently, we proposed a simple hypothesis that 4, 5-didehydro GGA induced apoptosis by way of down-regulation of TGFα gene expression and established the cells in the state of transcriptional deprivation of this trophic factor. We tested whether TGFα was really essential for HuH-7 cells to grow without apoptosis (Fig. 4A). In place of 4, 5-didehydro GGA, anti-TGFα monoclonal antibody was added to serum-free fresh medium. On the second day the cell number in the treated cells showed a dramatic decrease, whereas number of control cells increased. The same thing was true in treatment with anti-EGF receptor monoclonal antibody. The cytokilling effect of both monoclonal antibodies was shown to result in apoptosis proved by a characteristic genomic DNA ladder formation (Fig. 4B).

In this communication, we show that apoptosis of human hepatoma-derived cell line, HuH-7 induced by 4, 5-didehydro GGA is attributed to down-regulation of TGFα mRNA. It has been well documented that growth factor deprivation induces apoptosis, in general (12). To our knowledge, however, this is the first report that the mechanism of apoptosis induced by such a small lipid as 4, 5-didehydro GGA is suggested that the acid brings about down expression of peptide growth factor (TGFα in this case) and brings the cells into the same state of growth factor deprivation. The signal from TGFα is probably essential for the cells to survive, because when we withdrew autocrined TGFα by means of the neutralizing monoclonal antibody against TGFα, the cells no longer stayed at the substratum and died by apoptosis (Fig. 4A). Therefore, it is conceivable that

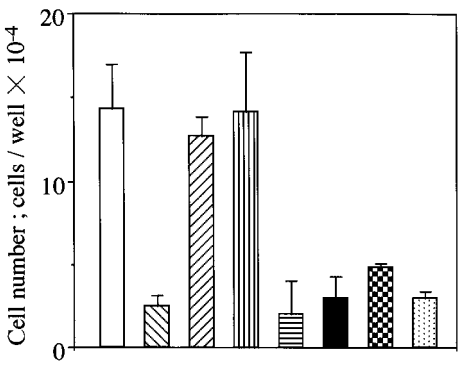

FIG. 2. Inhibition of 4, 5-didehydro GGA-induced apoptosis by TGFα or EGF. Viable cells were counted 8 hr after treatment with 4, 5-didehydro GGa and several hepatotrophic factors (▨; TGFα, ▥; EGF, ▤; Insulin, ■; IGF-II, ▩; T3, ▦; HGF) or with only 4, 5-didehydro GGA (▨) or with the vehicle alone □. Each point represents the mean ± S.E. (n = 3).

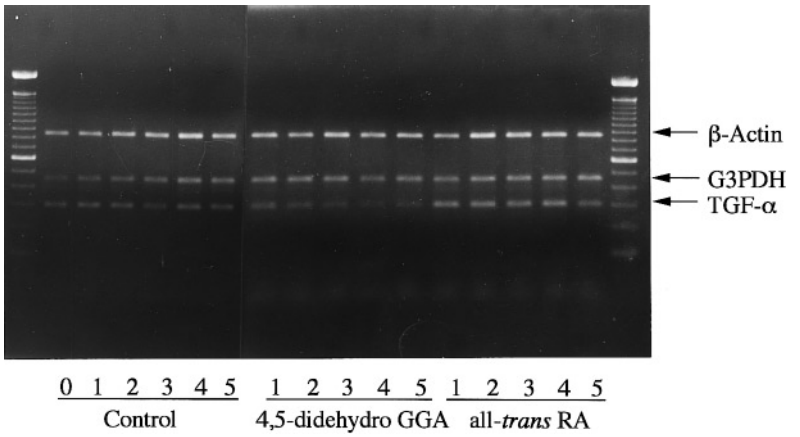

FIG. 3. Down-regulation of TGFα mRNA by 4, 5-didehydro GGA. TGFα mRNA levels were analyzed by RT–PCR described in *MATERIALS AND METHODS.* Each lane shows time of after treatment (lane 0:0 hr, 1:0.5 hr, 2:1 hr, 3:2 hr, 4:4 hr, 5:6 hr).

TGFα plays an important role in HuH-7 cells to commit themselves to proliferation or death. This gives us a very interesting idea for simultaneous understanding of mutually exclusive processes of carcinogenesis and apoptosis. Wyllie (13) had already proposed that cell states could be divided into three extreme states by the relationship between c-*myc* and growth factors; growth arrest (c-*myc* off and growth factor absent), population expansion (c-*myc* on, growth factor present), and apoptosis (c-*myc* on, growth factor off). We investigated the changes of c-*myc* expression in HuH-7 cells after 4, 5-didehydro GGA treatment. As shown in Fig. 5, the expression of c-*myc* gradually

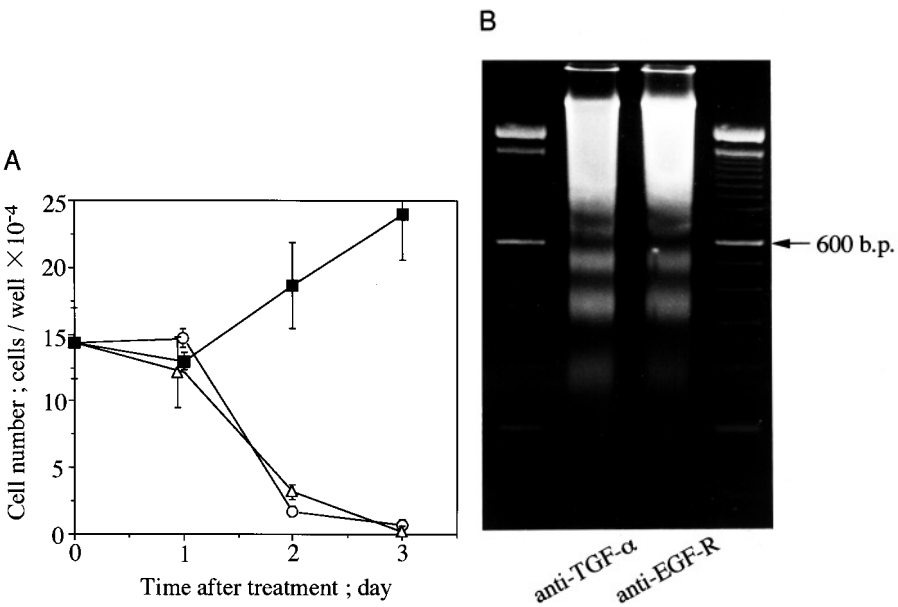

FIG. 4. (A)Induction of apoptosis by anti-TGFα or anti-EGF receptor monoclonal antibody. Time course of changes in cell numbers after treatment of anti-TGFα or anti-EGF receptor monoclonal antibody. Cells were treated with anti-TGFα (○) or anti-EGF receptor (△) monoclonal antibody or with the buffer alone (■). Each point represents the mean ± S.E. (n = 3). (B) Electrophoresis of genomic DNA from HuH-7 cells after treatment with anti-TGFα or anti-EGF receptor monoclonal antibody. Genomic DNA was extracted from the cells 2 days after the treatment with each monoclonal antibody and loaded onto a 1.0% agarose gel. The fragmented DNA was visualized with ethidium bromide staining.

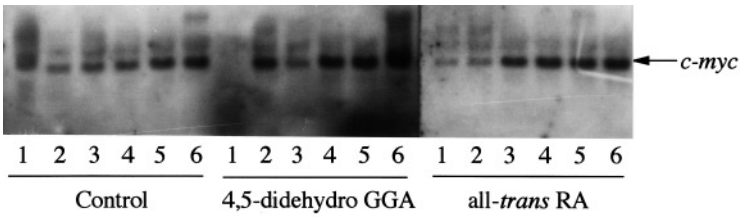

FIG. 5. Northern blot hybridization of c-*myc* mRNA from HuH-7 cells after treatment with 4, 5-didehydro GGA. Total RNA was isolated from non-treated or 10 μM all-*trans* RA-treated or 4, 5-didehydro GGA-treated cells at each point after treatment (lane 1: 0.5 hr, 2:1 hr, 3:2 hr, 4:3 hr, 5:4 hr, 6:6 hr).

increased 2 hr after the treatment and after 4 hr the expression reached almost plateau level and the same changes were observed in control cells as well as all-*trans* RA-treated cells. By taking Wyllie's words, the control and RA-treated cells were in the state of ''c-*myc* on, growth factor present'', so they increased in number, but the 4, 5-didehydro GGA-treated cells were in the state of ''c-*myc* on, growth factor off'' and fell in apoptosis. Therefore, the present results totally fit the aforementioned Wyllie's proposal for apoptosis.

Our data clearly show that 4, 5-didehydro GGA induced apoptosis in human hepatoma-derived cell line, HuH-7 through the destruction of autocrine loop consisting of TGFα and EGF receptor. From the point of this mechanism and taking account into consideration that normal hepatocytes were totally insensitive, it may be worth applying 4, 5-didehydro GGA as a chemopreventive agent for liver carcinogenesis. In fact, a phase II clinical trial for prevention of recurrence of hepatoma in patients after radical operation of hepatoma is proved effective(14).

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