INHIBITORY EFFECTS OF POLYPRENOIC ACID (E5166) ON PRODUCTION AND SECRETION OF α-FETOPROTEIN AND ON CELL KINETICS IN HUMAN HEPATOMA CELLS

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We investigated the effects of polyprenoic acid, E5166, on the production and secretion of α-fetoprotein (AFP) and on cell kinetics in a human hepatoma cell line (HuH-7). The cellular AFP content, measured flow cytometrically for cells stained by an indirect immunofluorescence method, was decreased by treatment with E5166. AFP in the culture medium decreased exponentially during exposure of cells to the drug. These changes were dose-dependent. The growth of HuH-7 cells in vitro was clearly suppressed in the presence of E5166. The inhibition of growth depended on the concentration of the agent. The fraction of S phase cells decreased relatively in the cells treated with a high concentration of the drug, whereas it increased in the cells treated with lower doses.

Key words: α-Fetoprotein — DNA — Hepatoma — Cell kinetics — Flow cytometry

Alpha-fetoprotein (AFP) is a major serum protein synthesized during the fetal stage, and it virtually disappears in the neonatal stage. It is well known that AFP is produced by hepatocellular carcinoma and is detectable in sera of patients with primary hepatoma. It has been observed that the tumor growth rate is correlated with the level of serum AFP.1 There is evidence that AFP production and release are closely linked to the cell cycle,2, 3) the cell proliferative activity1) and cellular differentiation.5, 6) Physiological regulation of cell proliferation appears to involve the coupling of growth inhibition and cellular differentiation.7) Vitamin A and its analogues are important regulators of differentiation and maintenance of normal epithelium.8) They have been shown to modulate cell proliferation and to have potential value as cancer-preventive or -therapeutic agents.9, 10) Recently, various vitamin A analogues have been synthesized for potential clinical application. One of the synthetic polyprenoic acid derivatives, all-trans-3, 7, 11, 15-tetramethyl-2, 4, 6, 10, 14-hexadecapentaenoic acid (E5166), has a strong binding affinity to cellular retinoic acid-binding protein11) and reduces the incidence of chemically induced tumors of the liver.12) However, little is known about the relevance of the modulation of cell growth by the polyprenoic acid to the expression of AFP gene in hepatoma cells. We present here data clarifying the effects of E5166 on AFP production and secretion, as well as their relation to cell kinetics in a human hepatoma cell line.

MATERIALS AND METHODS

Cell Culture HuH-7 cells established from human hepatocellular carcinoma13) were routinely cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal calf serum (Flow Lab., USA). The culture medium was replaced every day with fresh medium. The cultures were carried out in a fully humidified atmosphere containing 5% CO2. The cells which synthesized and secreted α-fetoprotein into the medium were passaged every 7 days by dispersion with Puck's solution.14) Cells in the exponential growth phase were used in this experiment.

Treatment with E5166 The cells were exposed to various concentrations of polyprenoic acid, E5166, for up to 4 days. The structural formula of the drug is shown in Fig. 1. It was generously
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given by Eisai Co., Ltd. (Tokyo). E5166 was freshly prepared immediately prior to use and care was exercised in shielding both the drug stock and cultures containing the drug from light. The drug was dissolved at a concentration of 7.5 mg/ml in 1 ml of DMSO and 3 ml of 100% ethanol, and then added to 55-cm² culture flasks (Falcon Plastic, USA) 48 hr after plating the cells, to give the desired concentrations. The medium for control cells contained the same concentrations of DMSO and ethanol as the treated medium. The medium was replaced every day by freshly prepared medium and all procedures were carried out in subdued light. At the indicated times, cells were removed from culture dishes by using Puck’s solution.14) Cell counts were performed on a Coulter counter (Coulter Electronics Inc., USA). Cell viability was determined in terms of PI exclusion.15)

Immunological Staining The cells were fixed in 70% ethanol overnight at 4°C, then they were washed three times in cold phosphate-buffered saline (PBS) and stained for cellular AFP by an indirect immunofluorescence method as previously described.3) Briefly, the cells were exposed for 1 to 2 hr to the anti-AFP antibody solution. The samples were washed twice in PBS, centrifuged, and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG antibody solution (Dako Co., Denmark). The antibodies used in this investigation were diluted to 1:50 with PBS containing 0.5% bovine serum albumin (Calbiochem, Co., USA) and 0.5% Tween 20 (Katayama Chemical Co., Osaka). As the control, the other set was exposed only to the fluorescent second antibody. Following incubation, each sample was rinsed in PBS and evaluated by FCM and with a fluorescence microscope. The procedures for immunological staining were performed at room temperature.

DNA Staining The cells harvested from the dishes were washed in cold PBS and centrifuged. The pelleted cells were suspended in 1 ml of PBS containing 0.5% Triton X-100 (Katayama Chemicals Co.) and 0.5% RNase (Sigma Chemicals Co., USA). Then, DNA staining was carried out with 50 μg/ml of propidium iodide (PI, Calbiochem. Co.).

Flow Cytometry The cell samples were sieved through a 43-μm nylon mesh filter to remove cell clusters prior to flow cytometric measurements. FITC fluorescence was measured using a FACS analyzer (Becton Dickinson Co., USA) in a linear scale mode at a wavelength of 520 nm with a 70 μm flow-cell at the cell stream. The gating window was set according to the particle size, so as to eliminate cell debris from the measurements. For determination of fluorescence intensity from PI, red fluorescence was collected through a 580-nm long-pass filter and recorded as measure of cellular content. The stained cells were excited with 485-nm radiation from a mercury arc lamp. Routinely, 1 × 10⁶ cells were measured and 256-channel histograms were generated. The instrument was calibrated using 10-μm fluorescent beads (Coulter Electronics Inc.); the coefficient of variation was less than 1.8. The analysis of DNA distribution histograms was done by computer.16)

Measurement of AFP in Medium A radioimmunoassay method was employed.

RESULTS

AFP Production and Secretion Green fluorescence was not detected in the control cells on the scale used for cells stained by the indirect immunofluorescence method. In contrast, the cells not exposed to polyprenoic acid, E5166, showed bright green fluorescence in their cytoplasm. Flow cytometric measurement revealed that individual cells have markedly different abilities to produce AFP. The fluorescence intensity slightly increased during the 4-day culture. On the other hand, the fluorescence intensity from the cells treated with the drug decreased markedly during culture and on day 4 the AFP content in the cells cultured in 20 μg/ml E5166 was only about 50% of that in the unexposed cells (Fig. 2). The reduction in AFP content was dependent on the dose of E5166.

In the case of the untreated cells, the amount of AFP in the culture medium, which was replaced every day by fresh medium, increased exponentially. However, the AFP content per cell in the medium remained almost constant throughout the experiment (Fig. 3). On the other hand, AFP in the

all trans -3, 7, 11, 15-tetramethyl-2, 4, 6, 10, 14-hexadecapentaenoic acid

Fig. 1. Chemical structure of polyprenoic acid (E5166). Molecular weight: 302.46.
medium of the cells treated with the drug decreased exponentially, and the AFP content per cell also decreased markedly (Fig. 4). The difference between the two was clearer on a semi-logarithmic scale, and the regression lines were as follows: \( Y=0.101X+1.874 \) (correlation efficient \( r=0.980 \)) for the culture with E5166 and \( Y=-0.26X+1.912 \) \( (r=0.999) \) for the culture without the agent.

**Cell Kinetics** The doubling time of HuH-7 cells was about 3 days under the standard culture conditions. However, the growth of cells exposed to 20 \( \mu g/ml \) E5166 ceased after the initial 24 hr (Fig. 4). As compared to the untreated cells, the cells exposed to the drug displayed an increase in the fraction of S phase and a decrease in G2M phase 24 hr after treatment with E5166, but subsequently the percentages of the three compartments (G1/0, S and G2M phases) of the cell cycle were similar to the control values (Fig. 5).

Low doses of E5166 resulted in an increase in the fraction of S phase cells, whereas the high dose caused a reduction of this fraction (Fig. 6). However, the growth suppression depended on the concentration of E5166.

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**Fig. 2.** The effect of E5166 on AFP production of HuH-7 cells. Treatment with 20 \( \mu g/ml \) E5166 led to a decrease in cellular AFP content which depended to some extent on the exposure time. AFP content was reduced to about a half of that in the control cells.

**Fig. 3.** Marked decrease in AFP secretion induced by E5166 treatment. AFP in the medium from the culture without E5166 increased exponentially (A ○) but the amount of AFP per 2.5 × 10⁶ cells remained constant (B ○). In contrast, the treatment with 20 \( \mu g/ml \) E5166 resulted in a decrease in AFP in the medium (A&B ●). The culture medium was replaced every day with fresh medium.
It is well known that vitamin A and its analogues promote differentiation and inhibit proliferation of many types of cells. Furthermore, there is a distinct cancer-preventive effect in experimental animals. Poly-prenoic acid, E5166, was also shown to inhibit the process of chemical carcinogenesis of the liver, and to induce HL-60 (human promyelocytic leukemia) cells to granulocytic differentiation (data not shown). For HL-60 cells treated with the drug the growth inhibition was accompanied by the accumulation of cells in the G1/0 phase of the cell cycle (data not shown). In HuH-7 cells exposed to 20 µg/ml E5166, however, the pattern of the DNA histogram did not differ from that in the untreated cells, except for a modest increase in the S phase fraction associated with a decrease in the G2M phase on day 1, although...
though cell multiplication was markedly inhibited after the 2nd day. We came to the conclusion that the cells progress very slowly through the cell cycle under the culture conditions mentioned above, since the number of cells did not increase even though the proportions of the compartments in the cell cycle were similar to the control values. However, it is likely that the cells in the G2 phase, when exposed to the drug, will progress through the cell cycle without significant disturbance of the cell kinetics during the initial 24 hr.

A high concentration of the drug markedly diminished the fraction of S phase cells, while lower concentrations increased the proportion of the fraction. The mechanisms of the growth inhibitory action of E5166 remain unclear, although the possibility of a direct effect on the cells cannot be completely ruled out. These observations may imply that E5166 leads in some instances to suppression of cell growth and in others to terminal differentiation. The differentiation induced by E5166 was accompanied by an accumulation of HL-60 cells in G1/0.

Slowly growing near-diploid rat hepatomas generally do not produce elevation of serum AFP when transplanted to syngeneic recipients, whereas fast-growing aneuploid tumors do.7) AFP is synthesized and then secreted into the medium by the hepatoma cells. The amount of AFP in both HuH-7 cells and their culture medium decreased precipitously soon after the treatment with E5166. It is likely that the release of AFP from the cells is affected by the drug more than the synthesis of AFP. With increase in the drug concentration and the exposure time, AFP production and release were reduced. Carcinoembryonic antigen content in the human rectal adenocarcinoma cell line decreased during culture in the presence of retinoic acid.17) Little information is available concerning mechanisms by which the polypropenoic acid regulates AFP gene transcription and controls cell proliferation. The program for the biological response of the target cell may reside in the receptors (such as retinoid binding protein) rather than in the drug itself. The rate of synthesis of AFP in the rat is correlated with cellular levels of its mRNA.18) It appears most likely that the polypropenoic acid exerts some fundamental control on gene expression. The drug may cause cells to regulate the gene expression by a mechanism similar to that in the case of steroid hormones or retinoids; a complex of E5166 and binding protein may modify gene transcription.19) The reduction of AFP appears to precede changes of cell proliferation, although AFP production and secretion are closely linked to the conditions of cell growth. Further studies should be made to clarify the relationship between cell differentiation and AFP synthesis in HuH-7 cells, but the present results indicate the potential usefulness of E5166 for chemotherapy of tumors.

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