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K252a: a new blocker of the cell-cycle at G1 phase in a human hepatoma cell line

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Abstract. The administration of 200 nM K252a to HuH7 suppressed the proliferation of the cells almost completely. The uptake of [^{3}H]thymidine was inhibited, and flow cytometry revealed only one peak at 2C on day 3 after treatment with 100 nM K252a. The expression of proto-oncogene c-myc was not reduced. Despite the blockage at G1, both the size of the cells and the amount of cell protein had increased by 4 times by day 3 after treatment with K252a, while the cells secreted albumin and α-fetoprotein into the medium as usual. These results show that K252a can increase the cell size of HuH7 without losing its function by blocking the cell cycle at G1 phase.

Key words. K252a; G1 block; cell size; c-myc; albumin; HuH7.

Introduction

Although it is rare for hepatocytes of adult animals to proliferate, they replicate readily in response to the loss of liver cells elicited by chemical injury or partial hepatectomy[1]. The level of α-fetoprotein in the blood increases as the liver regenerates. The hepatocyte and the epidermal growth factors are both considered to be hepatotrophic, and to trigger the regeneration of liver following a partial hepatectomy or liver injury[2]. However, the mechanism which triggers and regulates the various stages of compensatory growth in the liver is not yet understood clearly. Transduction of signals through the cell membrane involves a variety of enzymes that are implicated in the phosphorylation and dephosphorylation of many substrates.

K252a, a staurosporine-like indolocarbazole derivative isolated from the culture broth of *Nocardiopsis* sp.[3] was recently found to inhibit protein kinase C[4,5], cAMP-dependent protein kinase[4], cGMP-dependent protein kinase[4] and myosin light chain kinase[6] with a K_i of about 20 nM, and Ca^{++}/calmodulin-dependent protein kinase II with a K_i of 1.8 nM[7]. Recently, K252a was found to cause DNA re-replication in rat diploid fibroblasts without an intervening mitosis, thus producing tetraploid cells[8].

In this study, we report that K252a blocks the cell cycle in HuH7 human hepatoma cells at G1 after the expression of c-myc, and increases the cell size, without affecting the functioning of the cells.

Materials and methods

Materials. K252a (96.99% pure) was a gift from Kyowa Hakko, Co., Ltd. (Machida, Japan); [methyl-^{3}H]thymidine was purchased from ICN Biomedicals, Inc. (Irvin, USA), and aphidicolin was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Other chemicals used in this study were of reagent grade.

Cell culture. HuH7, a differentiated human hepatoma cell line (kindly supplied by Drs J. Sato and H. Nakabayashi, University of Okayama, Japan)[9]; Chang liver, a human liver cell line; Hep G2, a human hepatoma cell line; and HOS, a human osteosarcoma cell line, were cultured in an RPMI 1640 medium containing 1% fetal bovine serum or in Dulbecco's modified MEM containing 5% fetal bovine serum, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The number of cells was counted with a hemocytometer. Cell viability was tested by the trypan blue dye exclusion method.

Cell protein and DNA replication assay. The protein content of the cells was assayed in the trichloroacetic acid precipitable fraction by Lowry's method[10]. A volume of 2 μCi/ml [methyl-^{3}H]thymidine (0.3 mCi/μmole) was applied to cells cultured for 2 days in the absence or presence of various concentrations of K252a. After 6 h, the cells were lysed by adding NaOH, and the radioactivity was determined with a Packard liquid scintillation system, Tricarb 2200 (Packard Co., Chicago, USA). Net DNA replication was assayed by subtracting the [^{3}H]thymidine uptake of the dish treated with 2 μM aphidicolin from the uptake with each concentration of K252a. Albumin and α-fetoprotein were determined in the medium by a latex agglutination test (Eiken Chemical Co., Ltd., Tokyo, Japan) and an enzyme-immunoassay method (Abbot Co., Chicago, USA), respectively.

Flow cytometry. Flow cytometry was used to analyze the distribution of DNA content of HuH7 cells. Proce-

dures for the preparation and staining of nuclei were described previously[11,12]. Total fluorescence intensity was determined by quantitative flow cytometry using the Epics C system (Coulter Electronics Inc., Hialeah, USA).

c-myc mRNA analysis. Total cellular RNA was purified by a guanidium thiocyanate-phenol chloroform extraction method[13] and electrophoresed in formaldehyde-1% agarose gels, blotted onto nylon filters and hybridized with [^{32}P]-labelled c-myc. The c-myc probe used in the present study was 1.5 kb ClaI-EcoRI fragment containing the third exon of c-myc[14].

Results

We first examined the effect of K252a on the proliferation of the human liver cell lines. The number of HuH7 in the control culture increased about 6-fold by day 3 after plating. The application of K252a on day 1 after plating inhibited the proliferation of HuH7 in a dose-dependent manner as well as the proliferation of Chang liver and Hep G2 (fig. 1). Although there was no increase in the number of cells in the presence of 200 nM K252a, more than 90% of the cells were viable even at 400 nM K252a, when viability was tested by the trypan blue dye exclusion method, and the cells began to proliferate again after the removal of K252a from the culture medium, reaching more than three times the original number by day 3. The DNA synthesis of HuH7, measured by the uptake of [methyl-^{3}H]thymidine, was inhibited almost completely on day 2 following treatment with 200 nM K252a (fig. 1). That of Chang liver and Hep G2 was inhibited by 60% and 50% with 400 nM K252a, respectively. The DNA content analyses by flow cytometry showed only one peak at 2C (diploid) on day 3 after treatment of exponentially growing HuH7 with 100 nM K252a (fig. 2a, b). Similar patterns were observed when the cultures were synchronized at G0 prior to treatment with 200 nM K252a, by serum starvation (fig. 2c, d), or synchronized at S phase by exposure to 1.5 μM aphidicolin (fig. 2e, f). These results suggest that K252a blocks the cell-cycle of HuH7 at G0 or G1, not at the other phases.

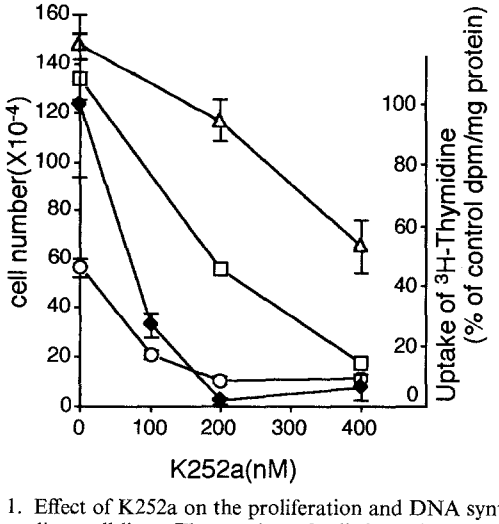

Figure 1. Effect of K252a on the proliferation and DNA synthesis of human liver cell lines. The number of cells in each human liver cell line was counted on day 3 after the addition of 0, 100, 200 and 400 nM K252a into the medium of the cultures seeded with 1×10^5 (HuH7, Chang liver) or 4×10^5 (Hep G2) cells/dish. Mean ± SE; n = 6. The uptake of [methyl-^{3}H]thymidine in HuH7 labeled for 6 h on day 2 after treatment with 0 (control), 100, 200 and 400 nM K252a was counted by liquid scintillation counter. Mean ± SE; n = 6. The mean of the controls (100%) was 18.1×10^3 dpm/mg protein. ○, HuH7 (cell number); □, Chang liver (cell number); △, Hep G2 (cell number); ◆, HuH7 (uptake of ^{3}H-thymidine).

The effect of K252a on the expression of proto-oncogene c-myc of HuH7 was analyzed because c-myc belongs to a set of the immediate early response genes whose expression is activated early during the G0 to G1 transition of cells[15]. K252a did not reduce the expression of c-myc on cells growing exponentially (fig. 3, A). K252a did not suppress the expression of c-myc on the cells at early G1 when quiescent cells were induced to proliferate by 1% FBS (fig. 3, B). Thus, K252a blocked the cell cycle of HuH7 at G1, but not at G0.

In addition to the G1 blockage, treatment of HuH7 with more than 200 nM K252a led to a marked increase in cell size. The diameter of the cells was elongated by 4 times that of the control cells. As the proliferation of cells was suppressed, the large cells were distributed separately, and they became rounder in shape, differing markedly from their normal morphology (fig. 4). The amount of protein in the cells on day 3 after treatment with 200 nM K252a was 4 times higher than that in the control cells (fig. 5). The functioning of the K252a-treated cells was investigated by the assay of albumin and α-fetoprotein secreted into the medium on day 3. The level of albumin in the medium was increased 3-fold per cell by 200 nM K252a as compared with the control cells, and the level of α-fetoprotein remained essentially unchanged when K252a was applied in doses ranging from 100 nM to 400 nM (fig. 6). Thus, the function of HuH7 was conserved even after K252a blocked the G1 phase of the cell-cycle.

Discussion

We showed that K252a blocked the cell-cycle of HuH7 at the G1 phase after the expression of proto-oncogene c-myc, which is strongly induced within the first 2 h of G1 by most growth stimuli[16] or after partial hepatectomy[17]. Since many proto-oncogenes are associated with the intracellular signal transduction of growth factors, screening for compounds that would block the cell-cycle at G1 has been carried out in the hope that they might be of use in anti-cancer therapy. W13[18], tunicamycin[19], sodium butyrate[20] and staurosporine[21] block the cell-cycle at G1. These substances affect calmodulin, transferase of sugar-chain, histone deacetylase and protein kinases, respectively. We observed that

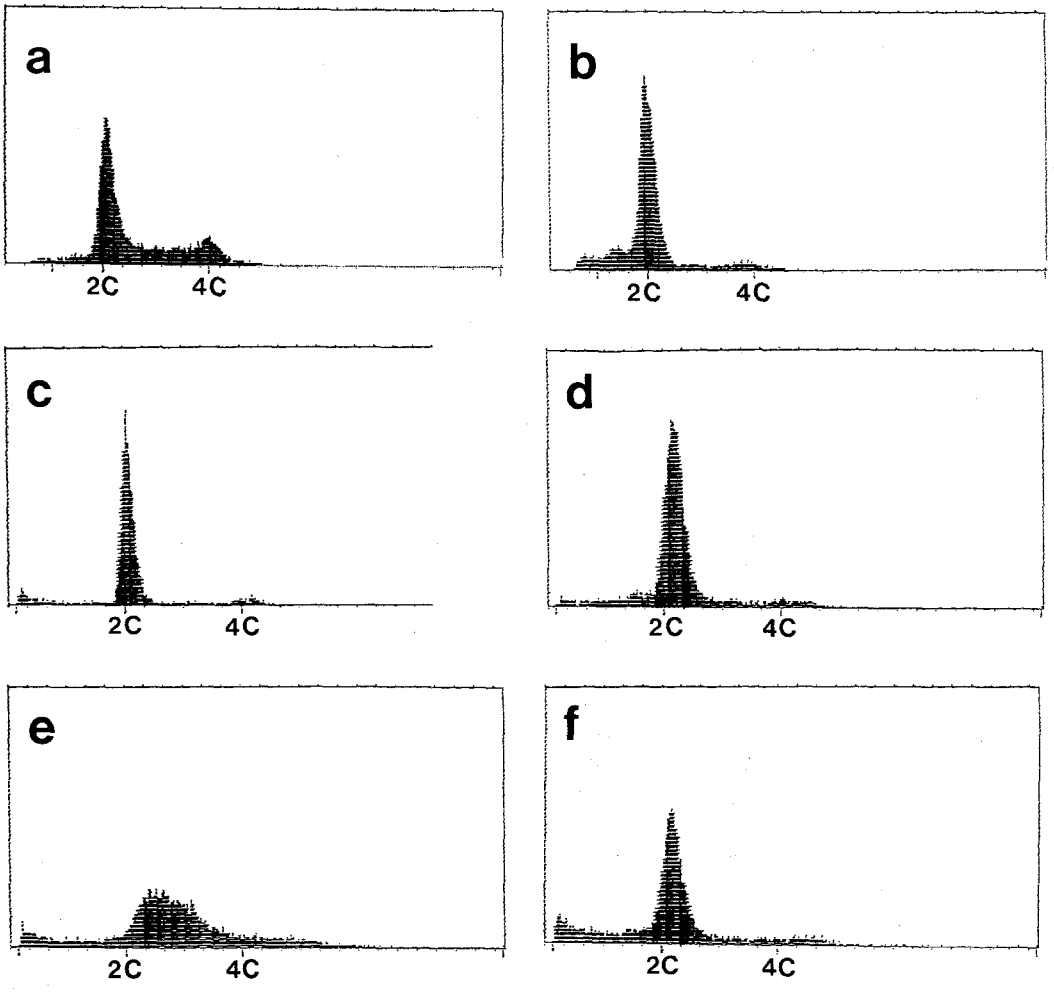

Figure 2. Analysis of DNA content of HuH7 by flow cytometry. *a* Exponentially growing cells; *b* cells on day 2 after treatment with 100 nM K252a; *c* quiescent cells in serum-free medium; *d* cells on day 2 after treatment with 200 nM K252a after the release from quiescence; *e* cells blocked at S phase by 1.5 μM aphidicolin, and *f* cells on day 2 after treatment with 200 nM K252a after the release from S phase block.

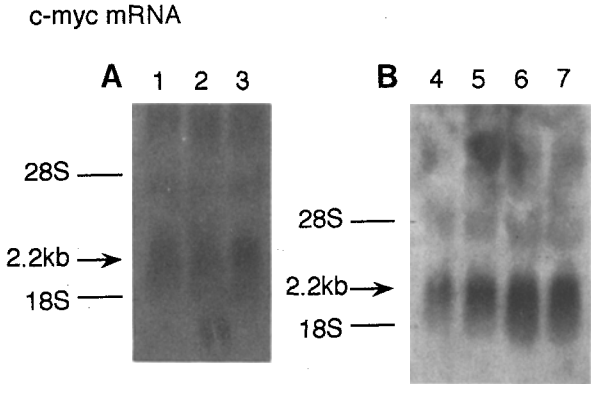

Figure 3. Effect of K252a on c-myc gene expression of HuH7. Arrows of 2.2 kb indicate c-myc mRNA. 20 μg of total cellular RNA purified from exponentially growing cells (A) and from synchronized cells at the quiescent phase deprived of serum for two days (B) were applied on each lane. Lane 1, control cells; lane 2, cells with 200 nM K252a; lane 3, cells with 400 nM K252a; lane 4, quiescent cells; lane 5, cells 1 h after the stimulation with 1% FBS; lane 6 and 7, cells 1 h after the stimulation with 1% FBS after the pre-treatment for 2 h with 200 nM K252a or with 400 nM K252a, respectively.

in addition to blocking the cell-cycle at G1, these compounds also suppressed the secretion of α-fetoprotein, and did not increase the size of the cell (data not shown). In contrast, K252a increased the cell size without affecting the main function of the cell, the secretion of α-fetoprotein into the medium. There was actually an increase of the secretion of albumin. Thus, K252a is an interesting G1 blocker different from those reported previously[18–21], and K252a may become a useful tool to study the growth and function of the cell.

It is not clear how K252a inhibits the cell-cycle of HuH7 at the G1 phase. Recently, we reported that K252a inhibited Ca^{++}/calmodulin-dependent protein kinase II with a K_i about a tenth less than that for other protein kinases[7]. The addition of serum to intact quiescent 3Y1 fibroblast cells rapidly elicited the autophosphorylation of Ca^{++}/calmodulin-dependent protein kinase II[22], indicating the activation of the enzyme. The indirect immunofluorescence study showed the participation of Ca^{++}/calmodulin-dependent protein kinase II during mitosis[23]. However, there is no report so far that Ca^{++}/

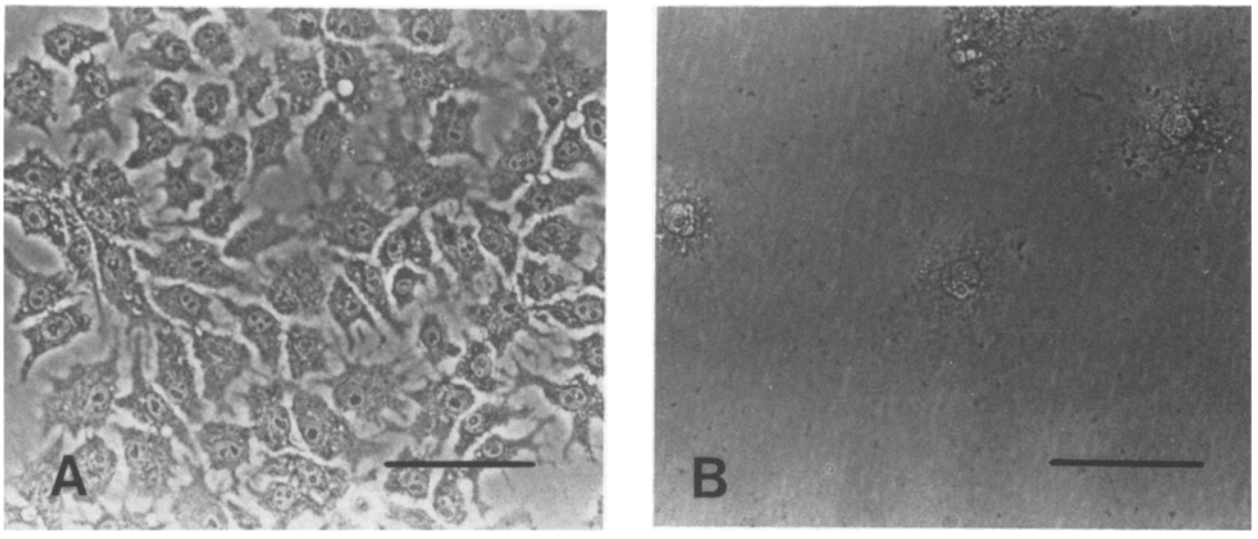

Figure 4. Effect of K252a on HuH7 morphology. *A* shows the control cells of HuH7 human hepatoma cell line without treatment. *B* shows the cells on day 3 after treatment with 200 nM K252a. Both groups of cells were stained with Giemsa after methanol fixation. The bar indicates 100 μm.

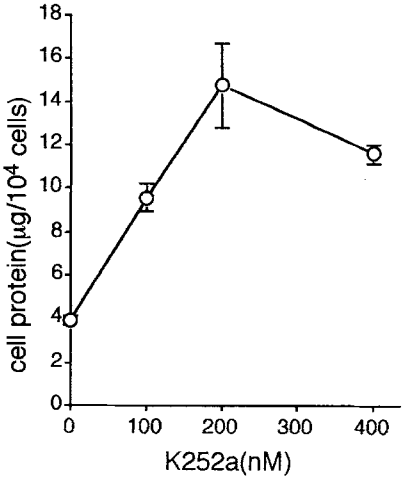

Figure 5. Effect of K252a on cell protein content of HuH7. Protein content was measured on day 3 after treatment with 0, 100, 200 and 400 nM K252a. Mean ± SE; n = 6.

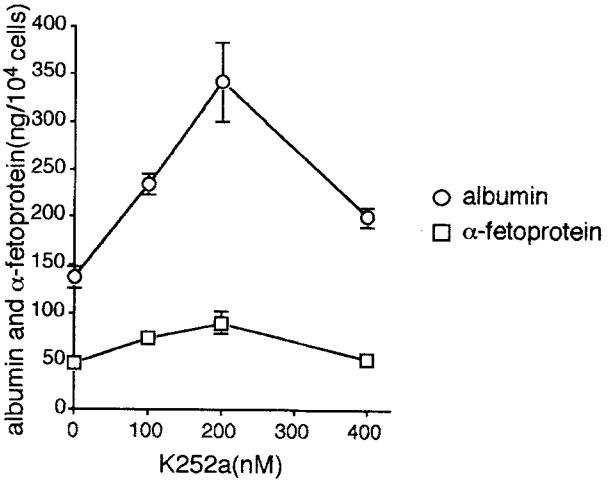

Figure 6. Effect of K252a on function of HuH7. Level of albumin and α-fetoprotein secreted into the medium was measured on day 3 after treatment with 0, 100, 200 and 400 nM K252a. (ng/10^4 cells). Mean ± SE; n = 3.

calmodulin-dependent protein kinase II plays an important role in the G1 phase. Ca^{++}/calmodulin-dependent protein kinase II is known to phosphorylate microtubule-associated proteins[24], including microtubule-associated protein 2 and τ-factor, whose phosphorylation affects microtubule assembly and the actin filament cross-linking activity. Although the development of elements of the cytoskeleton such as microfilaments or microtubules may be involved in the regulation of cell size, the mechanism that defines the cell size has not yet been fully elucidated.

It has been reported that K252a also inhibits mitogen-activated protein (MAP) kinase, but its 50% inhibitory concentration is much higher (0.05 μM) for MAP kinase[25] than that (0.0018 μM) for Ca^{++}/calmodulin-dependent protein kinase II[7]. Recently, Usui et al.[8] reported that K252a allowed cultured cells such as 3Y1 and SR-3Y1 (an RSV-transformed 3Y1 cell line) to by-pass mitosis and to re-replicate DNA with no effect on G1 phase. The difference between our results and theirs may be due to the difference between the cells used, because we also observed that K252a inhibited the uptake of [^{3}H]thymidine in Chang liver, Hep G2 and vascular smooth muscle cells[26], whereas it did not suppress that in HOS (data not shown). Future studies should define the specific sites and/or the regulatory proteins of the cell cycle which are affected by K252a.

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