INHIBITION OF c-*myc* GENE EXPRESSION IN MURINE LYMPHOBLASTOMA CELLS BY GELDANAMYCIN AND HERBIMYCIN, ANTIBIOTICS OF BENZOQUINOID ANSAMYCIN GROUP

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We have shown that geldanamycin (GDM), an antibiotic of benzoquinoid ansamycin group, inhibits DNA replication in cultured mouse lymphoblastoma L5178Y cells. Here we report that GDM selectively inhibited the expression of c-*myc* gene, proto-oncogene, along with suppression of DNA replication in L5178Y cells, which are consistent with our previous results that c-*myc* protein promotes cellular DNA replication. The significantly enhanced inhibition by GDM of DNA replication was observed, when the antibiotic was introduced at G1 stage prior to S phase of cell cycle. The results are in favor of the prospects that GDM inhibits DNA replication mainly at time of initiation, and that c-*myc* protein is essential for the initiation of cellular DNA replication. Furthermore, when c-*myc* expression was inhibited by GDM, the expression of p53 gene, the product of which may be another DNA replication protein, was stimulated in the tumor cells. Thus, GDM should be useful to investigate the molecular mechanism of DNA replication promoted by c-*myc* protein and also to distinguish the function of c-*myc* protein from that of p53 protein in DNA replication.

In recent years, the screening program of anticancer agents has been addressed to searching for inhibitors of oncogene function. Some antibiotics which inhibit oncogene function including genestein[1] and erbstatin[2] have been isolated under the program. It is worth asking as to whether the agent active against tumor cells inhibits the oncogene function or the expression of oncogene. We have previously reported that geldanamycin (GDM) which is active against eukaryotic cells including fungi, protozoa and tumor cells[3] preferentially inhibits DNA replication in cultured tumor cells[4,5]. Our results have shown that the antibiotic profoundly blocks the progression from G1 to S phase of cell cycle followed by the inhibition of DNA replication in cultured mouse lymphoblastoma L5178Y cells. These results lead us to the possibility that the initiation of DNA replication is a target of GDM. We have recently shown that c-*myc* protein localized in nuclei is functioning as an initiator of DNA replication in mouse and human cells[6,7]. To improve our understanding the mode of action and antitumor activity of the antibiotic, we asked whether GDM inhibits the function of c-*myc* protein or the expression of c-*myc* gene. Here we show the specific suppression of c-*myc* gene expression in mouse lymphoblastoma L5178Y cells by the antibiotic.

Materials and Methods

Chemicals

[^{3}H]Thymidine (28 Ci/mmol) and deoxycytidine 5′-[α-^{32}P]triphosphate (3,000 Ci/mmol) were purchased from New England Nuclear Co., Ltd., MA. GDM and herbimycin A (HBM) were gen-

erously provided by Dr. N. ŌTAKE, University of Teikyo, Tokyo, and Dr. S. ŌMURA, Institute of Kitasato, Tokyo, respectively. Ansamitosin P-3 (ASM P-3) was kindly supplied by Takeda Chemical Industries, Ltd., Osaka.

Cells

Mouse lymphoblastoma L5178Y cells were cultured in RPMI 1640 supplemented with 10% horse serum.

Pulse Labeling of Cells with [^{3}H]Thymidine

5×10^4 cells/ml of L5178Y cells were labeled with [^{3}H]thymidine (0.1 μCi/ml) for 1 hour. After labeling the cells were trapped on glass fiber disk (GF/C, Whattman), washed once with phosphate-buffered saline (PBS), twice with cold 5% trichloroacetic acid (TCA) and once with 1% acetic acid. The radioactivity incorporated into cells was counted in a scintillation counter.

Synchronization of L5178Y Cells

The cells were synchronized by the treatment with colcemid as described previously[8]. The cells arrested at metaphase divided within 1.5 hours after removal of colcemid, and enter S phase around 2 hours.

Hybridization of RNA with Gene Probes

The cultured L5178Y cells (5×10^5 cells/ml) of 30 ml culture were collected and washed once with PBS containing 2 mM EDTA. Total RNA was prepared by the guanidine-cesium chloride method as described previously[9]. The RNA was separated on 1.4% agarose gel containing formamide, transferred to nitrocellulose filter, and hybridized with ^{32}P-labeled gene probes according to the procedure described previously[10].

Results

Effect of GDM on DNA Replication in L5178Y Cells

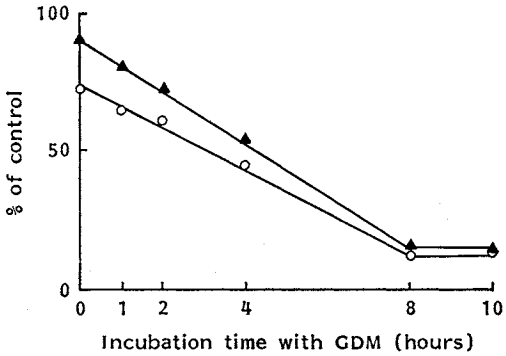
We have previously shown that GDM inhibits preferentially DNA synthesis, but not overall protein and RNA syntheses in cultured L5178Y cells[4,5]. Furthermore, the inhibition of DNA synthesis by the antibiotic was greater at high rates of DNA replication, suggesting that the antibiotic selectively inhibits initiation of DNA replication[4,5]. The rate of DNA replication in random cultured L5178Y cells was gradually decreased for up to about 8 hours (the time assumed to correspond with the spun of S phase of cell cycle) in the presence of the antibiotic as demonstrated in Fig. 1, suggesting that the antibiotic interrupts the progression from G1 to S phase of cell cycle. This speculation was supported by the following experiment. Significantly enhanced inhibition by GDM of DNA replication was observed, when the antibiotic was introduced at G1 prior to the S phase of the cell cycle as presented in Fig. 2. These results led us to postrate that the expression of the initiator protein for DNA replication is suppressed in the presence of GDM.

Inhibition of c-*myc* Gene Expression by GDM

Our recent findings indicate that c-*myc* protein localized in nuclei is functioning as an initiator of DNA replication in mammalian cells[6,7]. Our concern has been focused on whether the antibiotic inhibits the function of c-*myc* protein or the expression of c-*myc* gene leading to the inhibition of DNA replication in L5178Y cells. The effect of GDM on gene expression of c-*myc* was investigated. The RNA transcripts of c-*myc* in L5178Y cells treated with GDM for 8 hours were markedly decreased (Fig. 3A and B), whereas no reduction of RNA transcripts of hypoxanthine phosphoribosyltransferase (HPRT) gene which is the house keeping gene was detected in the presence of GDM (Fig. 3C). Although these results clearly indicate that GDM inhibits c-*myc* gene expression, it is possible that

the inhibition of c-*myc* gene expression by GDM results from suppression of DNA replication. In an attempt to clarify this possibility, we examined the effect of hydroxyurea (HU) on the c-*myc* gene expression. HU, an inhibitor of ribonucleotide reductase which inhibits the progression from G1 to S phase of cell cycle by decreasing the level of deoxyribonucleotide pools and results in inhibition of DNA replication. HU did not

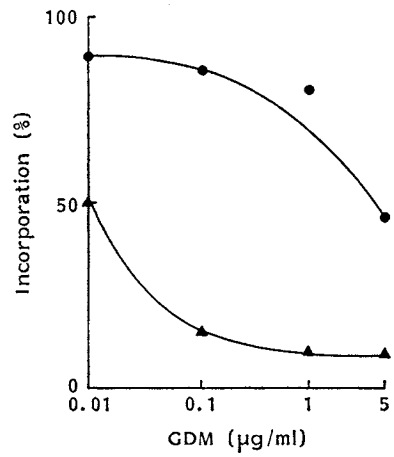
Fig. 1. Inhibition of DNA replication in L5178Y cells by GDM.



L5178Y cells were preincubated with GDM at the concentration of 1 μg/ml (○), and 0.1 μg/ml (▲), respectively, for up to 10 hours as specified, and then pulse labeled with 0.1 μCi/ml of [^{3}H]-thymidine for 1 hour. The degree of incorporation per 5×10^4 cells expressed by percentage of control corresponds to the rate of DNA replication. The [^{3}H]thymidine incorporation of the control was 1,200 dmp per 5×10^4 cells.

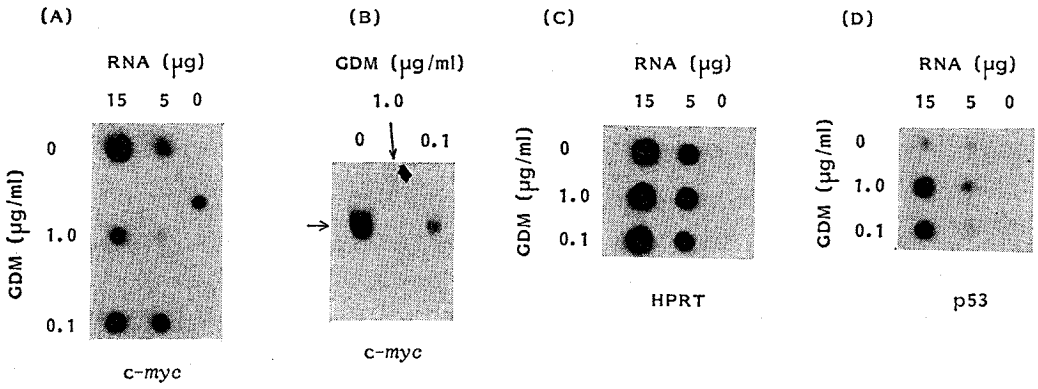
Fig. 2. Effect of GDM on DNA replication in the random culture or the synchronized culture at G1 phase of cell cycle in L5178Y cells.

● Random culture, ▲ synchronized culture at G1 phase.



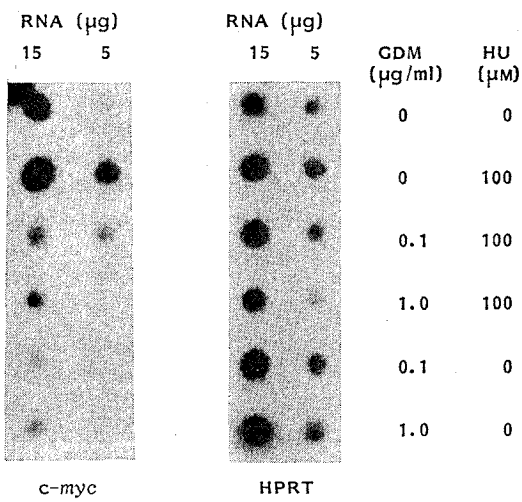
L5178Y cells of random or synchronized at G1 phase as described[7] at the cell density of 5×10^4 per ml were preincubated with GDM for 4 hours and pulse labeled with 1 μCi/ml of [^{3}H]thymidine for 1 hour. The degree of incorporation was expressed by percentage of control. The [^{3}H]thymidine incorporation of the control were 12,000 dpm for random culture and 5,000 dpm for synchronized culture per 5×10^4 cells, respectively.

Fig. 3. Inhibition of c-*myc* gene expression by GDM in L5178Y cells in the presence of hydroxyurea.



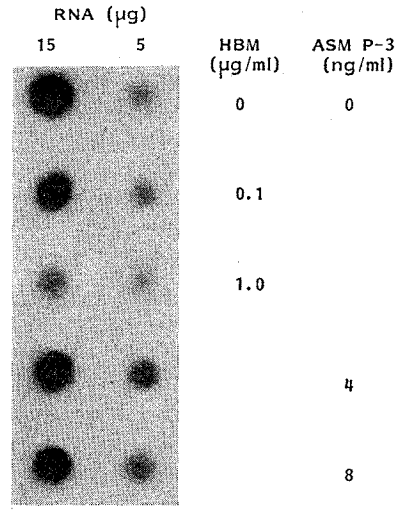
The RNA extracted from L5178Y cells was hybridized with gene probes including c-*myc* (A, B), HPRT (C) and p53 (D). The hybridization was carried out by dot-blotting (A, C, D) or northern technique (B) (20 μg of total RNA was blotted on filter), and then the amount of transcripts was detected by autoradiography. L5178Y cells were treated with GDM for 8 hours before RNA extraction.

Fig. 4. Inhibition of c-*myc* expression by GDM in L5178Y cells.



The amount of RNA transcripts was detected by dot-blotting hybridization for c-*myc* (left), and for HPRT (right).

Fig. 5. Effect of HBM and ASM P-3 on c-*myc* gene expression.



The amount of c-*myc* transcripts was detected by dot-blotting hybridization. The concentrations of ASM P-3 were enough to inhibit the cell growth.

affect the c-*myc* expression as previously reported[11]. The simultaneous addition of HU and GDM to the cultured L5178Y cells suppressed the c-*myc* gene expression and also affected HPRT expression to the same degree as GDM alone (Fig. 4), indicating that the inhibition of c-*myc* gene expression by GDM is not influenced by suppression of DNA replication. These results suggest that GDM inhibits the specific transcription process for c-*myc* gene expression in a direct or an indirect manner. Specific inhibition of c-*myc* gene expression by the antibiotic was also found in human leukemia HL60 cells (data not shown).

Stimulation of p53 Gene Expression by GDM

The gene expression of p53 was apparently stimulated in the presence of GDM (Fig. 3D). The reason for the enhanced gene expression of p53 remains obscure at present. We have previously shown that p53 is also a DNA replication protein and that p53 recognizes a different origin of DNA replication from that of c-*myc* protein[12]. So, it is possible that p53 was overexpressed by a SOS function in the presence of GDM given the defect of DNA replication.

Effect of Other Antibiotics on c-*myc* Expression

The suppression of c-*myc* gene was also examined using other antibiotics, HBM which is a benzoquinoid ansamycin (BQA) antibiotic, and ASM P-3 which is structurally analogous to BQA, but exhibits a different mode of action from BQA interfering with microtuble assembly followed by mitosis inhibition[13]. As shown in Fig. 5, HBM inhibited the expression of c-*myc* gene, but ASM P-3 did not. Structural relationship among GDM, HBM and ASM P-3 will be discussed later.

Discussion

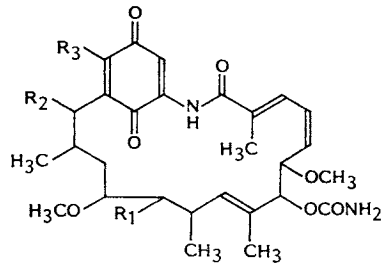
The studies in this manuscript show that the mode of inhibition of DNA replication by GDM is due to the suppression of c-*myc* gene expression, which is based on our previous report that c-*myc*

protein may be involved in the initiation of cellular DNA replication[6,7]. It is of our particular interest that GDM inhibits selectively c-*myc* gene expression, accompanied by the enhancement of p53 gene expression. It is possible that the p53 gene, the product of which is likely involved in DNA replication[12] became overexpressed to exert a SOS function following the defect of DNA replication, which resulted from inhibition of c-*myc* expression. Vast numbers of report have appeared that c-*myc* gene is overexpressed in cancer cells. So it is reasonable that antitumor activity of GDM is due to the suppression of c-*myc* gene expression followed by the inhibition of DNA replication.

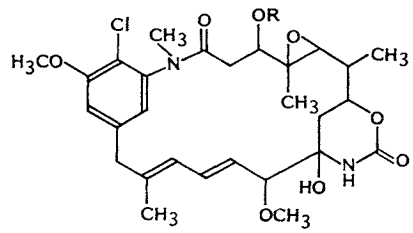
The multiplication of cells is often induced by the signals, such as mitogen, onto cell surface leading the activation of proto-oncogenes. The constitutive expression of *src* or *ras* in cancer cells alters the cellular metabolism and then the cells move to proliferation. The expression of oncogene on cell surface induces the enhancement of phosphatidylinositol metabolism followed by high level of Ca-cation influx and then activation of protein kinase C (PKC)[14]. Several lines of evidence indicate that PKC is involved in the signal transduction from cell surface to nuclei, during which the target protein(s) is phosphorylated, leading DNA replication. These lines of evidence lead us to have several possibilities concerning the mechanism of inhibition of c-*myc* gene expression by the antibiotic: The antibiotic might block the movement of signal of cell surface for activation of oncogene. The inhibition of activation of oncogenes on cell surface might result in alteration of PKC activity followed by c-*myc* gene expression. This possibility may be supported by previous results that the antibiotic reduced the tyrosine phosphorylation of *src* gene product in v-*src* transformed cells[15]. It is also possible that the antibiotic directly inhibits the PKC activity or the c-*myc* transcription. The c-*myc* protein is required both for the initiation of DNA replication and the c-*myc* transcription itself, which suggests that the initiation of DNA replication and c-*myc* expression are coordinately regulated. And it was also reported that the binding sites of c-*myc* protein shares the sequences necessary for the initiation of DNA replication and enhancer of c-*myc* transcription[16]. Thus, the inactivation of c-*myc* protein or its expression should be followed by the inhibition of initiation of DNA replication. It is likely that the mode of inhibition of DNA replication by GDM is the primary inhibition of c-*myc* transcription. The blockade of c-*myc* expression by the antibiotic will give rise to the accelerated inhibition of c-*myc* gene expression because c-*myc* protein itself may be necessary for c-*myc* gene expression as we suggested[16].

Although the mechanism of action of naphthoquinoid ansamycins, *i.e.* rifampicin and streptovaricins, has been extensively studied, and these antibiotics have become known as specific inhibitor of RNA synthesis in prokaryote interacting with RNA polymerase[17], the molecular mechanism of action of BQA antibiotics remains to be determined. The mode of action of benzenoid ansamycins which do not contain benzoquinoid ring, *i.e.* ASM P-3[13] and maytansine[18] has been revealed to inhibit mitosis by interacting with tublin molecule. The structural differences among GDM, HBM and ASM P-3 are shown in Fig. 6. We presented here the differences of the mode of action between these two groups of antibiotic on the c-*myc* expression in tumor cells. It has been reported that some inhibitors of c-*myc* expression including tumor necrosis factor[19], human interferon[20], neplanocin A[21], antipain[22], 2-aminopurine[23] and dimethyl sulfoxide[11] induce the differentiation of cells. However,

Fig. 6. The structures of GDM, HBM and ASM P-3.



GDM R_1=OH, R_2=H, R_3=OCH_3
HBMA R_1=R_2=OCH_3, R_3=H



ASM P-3 R=$COCH(CH_3)_2$

GDM did not induce the differentiation of HL-60 cells. The results indicate that the inhibition of c-*myc* gene expression is not always necessary for differentiation of cells.

Molecular mechanisms of DNA replication or differentiation of cells in eukaryote remain to be solved. To solve these, there exist possible strategies including *in vitro* system, mutational analysis *in vivo*, cloning of gene of interesting, and so on[24]. It is also useful to use inhibitors that work against DNA replication[25]. Our study presented here should provide a basis for understanding the regulation of DNA replication and the function of proto-oncogene products in regulation of cell proliferation in eukaryotes.

Acknowledgments

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