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EFFECT OF GENISTEIN ON TOPOISOMERASE ACTIVITY AND ON THE GROWTH OF [VAL 12]Ha-ras-TRANSFORMED NIH 3T3 CELLS

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SUMMARY: Genistein inhibited topoisomerase II and I; it increased the enzyme-DNA complex in L1210 cells at 1μg/ml, and interfered with pBR322 DNA relaxation by the enzymes. To test the role of topoisomerase in the transformation by oncogenes, the effect of genistein on the transformation of NIH 3T3 cells by transfection with [Val 12]Ha-ras was compared with that of N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK), since genistein inhibits tyrosine kinase as well as TLCK. Genistein reduced the number of foci of the transformed cells, and suppressed selectively the growth of ras-transformed NIH 3T3 cells but not normal NIH 3T3 cells. In contrast, TLCK did not affect the transformation. It inhibited the growth of the normal cells but not the transformed cells.

Topoisomerases are the enzymes introducing transient breaks in DNA backbone. They have been reported to participate in a variety of genetic processes such as replication (1-3), transcription (1-3), recombination (4-5), integration (6), and transposition (3). Our current interest in topoisomerases was whether they were also related to the transformation by ras-oncogenes. In order to examine this hypothesis a topoisomerase inhibitor would be useful. However, well known topoisomerase inhibitors such as adriamycin, 4'-(9-acridinylamino)methanesulfon-m-anisidine, and etoposide were unsuitable for this purpose because they killed normal NIH 3T3 cells used in transformation experiments at concentrations lower than those effective in inhibiting topoisomerase II.

In the course of the screening for topoisomerase inhibitors, we found that an isoflavonoid genistein was a topoisomerase inhibitor and its cytotoxicity toward normal cells was very weak; accordingly the role of topoisomerase in the transformation by oncogenic ras was subsequently investigated

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Abbreviation: TLCK, N-α-tosyl-L-lysyl-chloromethyl ketone.

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using genistein. Since genistein has been reported to inhibit tyrosine-specific protein kinase (7), the effect of a tyrosine kinase inhibitor, N-α-tosyl-L-lysyl-chloromethyl ketone was also compared with that of genistein. The results are presented in this communication.

MATERIALS AND METHODS

DNA and Cells A plasmid containing [Val 12]Ha-ras (pEJ) and NIH 3T3 cells transformed by transfection with pEJ (EJ cells) were kindly given by Dr. Gibbs of Merck Sharp and Dohme Research Laboratories (8). NIH 3T3 (ATCC, CRL1658) and L1210 cells (ATCC, CCL219) were purchased from Flow Labs (VA, USA). EJ and NIH 3T3 cells were cultured with Dulbecco's modified eagle medium containing 10% fetal bovine serum. L1210 cells were propagated using RPMI1640 medium supplemented with 10% fetal bovine serum.

Topoisomerases L1210 DNA topoisomerase II and I were partly purified with hydroxylapatite, phosphocellulose, and DNA cellulose column chromatography according to the procedures reported by Miller et. al. (9). The topoisomerase II preparation used at the present study was inactive in the absence of ATP.

Reagents Genistein was purified from the hydrolysate of soy bean meal which contained genistin (10). N-α-tosyl-L-lysyl chloromethyl ketone (TLCK) and 5-[125I]iodo-2'-deoxyuridine were commercial products of Sigma (MO, USA) and Amersham (Amersham UK), respectively.

Topoisomerase assays To determine the amount of the covalent enzyme-DNA complex in cells, the K/SDS precipitation method of Trask et. al. (11) was modified by Dr. Jones of Merck Sharp and Dohme Research Laboratories and further improved as follows. L1210, NIH 3T3 and EJ cells were prelabeled with 0.5uCi/ml 5-[125I]iodo-2'-deoxyuridine for 4hrs and then incubated with samples at 37°C for 40min. After the incubation, the cells were separated from the medium and lysed with lysis buffer (1.5% SDS, 5mM EDTA) at 65°C. The DNA in the lysate was sheared by pipetting it up and down 30 times with C20 micropipette tips (Gilson), and added a fourth volume of 325mM KCl. The K/SDS precipitates formed on ice were collected centrifugally, and dissolved in wash buffer (10mM Tris pH 8.0, 100mM KCl, 1mM EDTA) at 65°C. Following cooling on ice and recentrifugation, the radioactivity in the precipitate was measured with a γ-counter. DNA relaxation assay was performed with the method described by Miller et. al.(9). pBR322 DNA and the enzymes were incubated with samples at 37°C for 15min. After the incubation, the reaction mixture was treated with SDS, and submitted to 1% agarose gel electrophoresis.

Transformation assay Transformation of NIH 3T3 cells was done with calcium phosphate-DNA transfection method (12). NIH 3T3 cells plated into 60mm dishes were transfected with the plasmid pEJ. The cells were cultured without a sample for the first 3 days, and cultured further with a sample for 16 days. The medium was changed every 2 days. After the culture the cells were stained with Giemsa's solution and the number of foci was counted under a magnifier.

Cytotoxicity assay NIH 3T3 or EJ cells plated into wells of 48-well test plates were exposed to a sample for 14 days. The medium was replaced every 2 days with the same fresh medium. Effect on the cell growth was determined by cell count using a Coulter Counter.

RESULTS

Effect on covalent complex formation Genistein increased dose-dependently the amount of covalent complex between protein and DNA in L1210 cells at a range from 1 to 100μg/ml (Figure 1). The pattern of the dose response curve for genistein was similar to that of a nonintercalative topoisomerase inhibitor, etoposide. The curve differed from that of the DNA intercalater

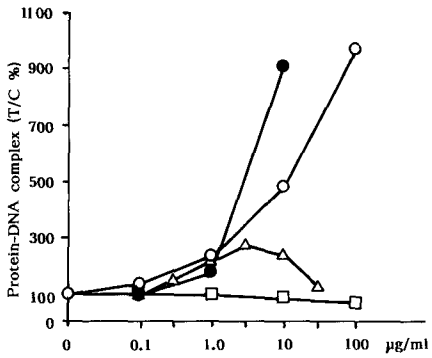

Figure 1. Effect of genistein on protein-DNA complex formation in L1210 cells Genistein(○), Etoposide(●), Daunomycin(△), TLCK(□). Cells prelabeled with 5-[125 I]iodo-2'-deoxyuridine were exposed to samples at 37°C for 40min.

daunomycin. Adriamycin was less effective in increasing the amount of the protein-DNA complex (not shown). Whereas TLCK did not affect the covalent complex formation in L1210 cells at concentrations from 1 up to 100μg/ml.

The effect of genistein on the complex formation in NIH 3T3 and EJ cells was almost the same as that in L1210 cells. At a concentration of 10μg/ml, it increased the amount of the complex in NIH 3T3 and EJ cells by 399 and 505%, respectively. However it was ineffective at 1μg/ml.

Effect on pBR322 DNA relaxation Genistein inhibited the relaxation of pBR322 DNA by topoisomerase II at concentrations more than 2μg/ml (Figure 2). Although etoposide specifically inhibited topoisomerase II, genistein also interfered with DNA relaxation catalyzed by topoisomerase I. Genistein did not change the super coiled structure of pBR322 DNA in the absence of the enzymes.

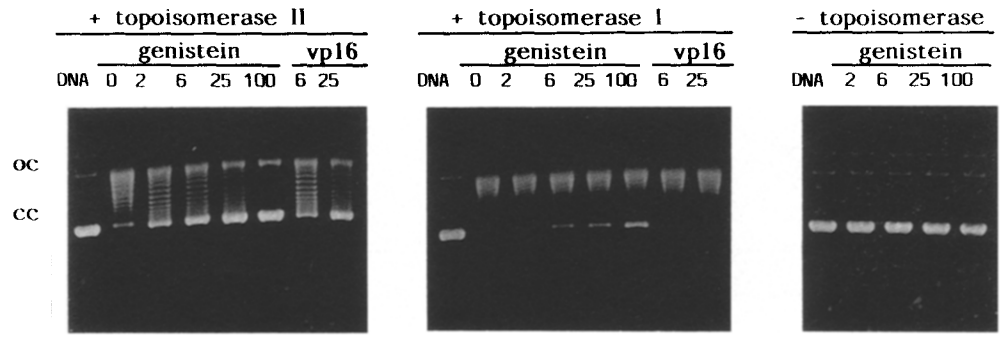

Figure 2. Effect of genistein on the relaxation of pBR322 by topoisomerases. pBR322 DNA was incubated with samples in the presence or absence of the enzymes at 37°C for 15min. Numbers in Figure 2 indicate sample concentratios (μg/ml).

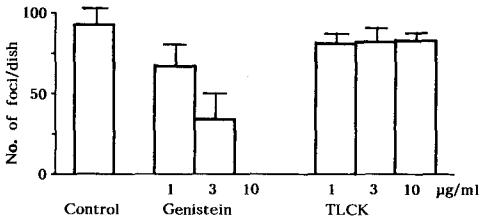

Figure 3. Effect of genistein and N-α-tosyl-L-lysyl-chloromethyl ketone on the transformation of NIH 3T3 cells transfected with a plasmid containing [Val 12]Ha-ras. Cells plated into 60mm dishes were transfected with the plasmid by calcium phosphate-DNA transfection method. They were cultured in the absence of samples for 3 days, and then in the presence of them for 16 days.

Effect on the transformation of NIH 3T3 cells by oncogenic ras Genistein was added to the culture medium from 3 days after the transfection. It reduced the number of foci of cells transformed by transfection with oncogenic Ha-ras. At 10μg/ml, no foci were counted (Figure 3). Since genistein also inhibit tyrosine-specific protein kinase (7), the effect of its inhibitor TLCK was tested. TLCK did not inhibit the transformation at concentrations less than 10μg/ml.

Effect on cell growth Genistein selectively inhibited the growth of EJ but not normal NIH 3T3 cells at 10μg/ml when it was added to the culture longer than 10 days (Figure 4). NIH 3T3 cells ceased their growth when exposed to 10μg/ml genistein, however they started to proliferate 4 days after the beginning of incubation with the agent, and their growth overtook almost that

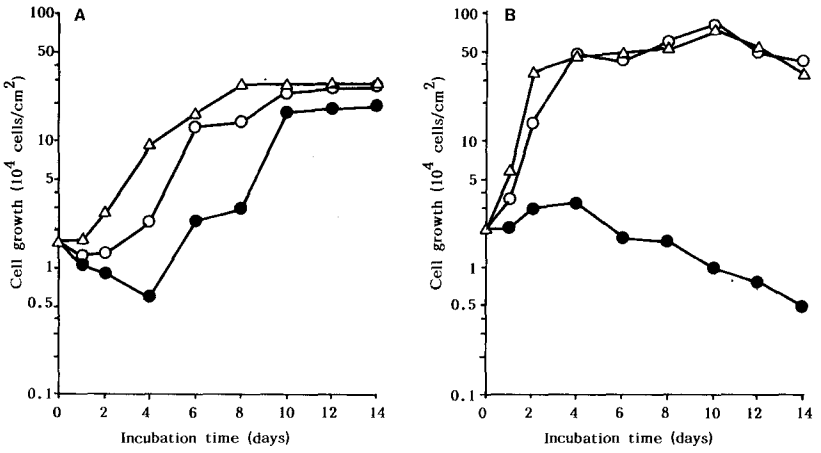

Figure 4. Effect of genistein on the growth of normal and [Val 12]Ha-ras-transformed NIH3T3 (EJ) cells. (A); normal NIH 3T3 cells. (B); EJ cells. Cells were exposed to genistein from day 0 through day 14. Cell growth was monitered by cell count using a Coulter counter. Genistein 0μg/ml(△), 3μg/ml (○), 10μg/ml(●).

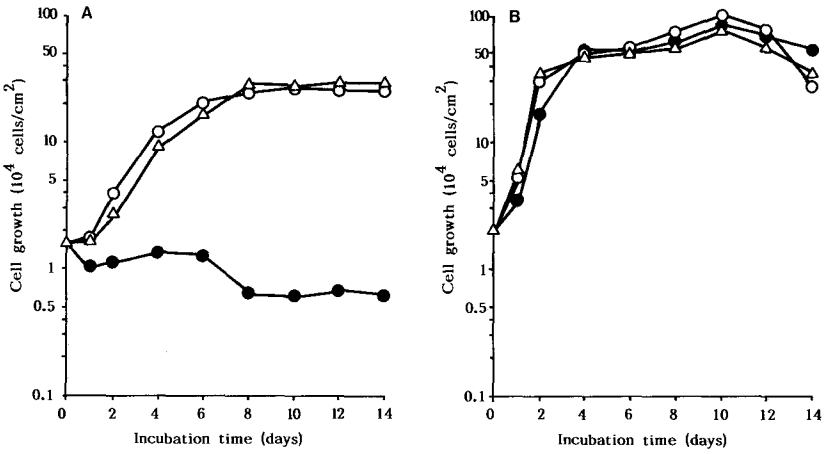

Figure 5. Effect of N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK) on the growth of normal and [Val 12]Ha-ras-transformed NIH3T3 (EJ) cells. (A); normal NIH 3T3 cells. (B); EJ cells. Cells were exposed to TLCK from day 0 through day 14. Cell growth was monitered by cell count using a Coulter counter. TLCK 0μg/ml(△), 10μg/ml(○), 30μg/ml(●).

of non treated cells 6 days thereafter. According to morphological investigations, no dead cells were observed among cells treated with genistein. The growth suppressing effect of genistein depended on the health of cells. When NIH 3T3 cells showed a tendency to spontaneously transform, genistein also inhibited the growth of such cells. In contrast, TLCK was toxic against the normal cells at 30μg/ml but not toxic against the transformed cells (Figure 5).

DISCUSSION

A large number of antitumor agents such as anthracycline antibiotics, etoposide, and 4'-(9-acridinylamino)-methanesulfon-m-anisidide are known to stabilize a cleavable topoisomerase II-DNA complex. Genistein increased the the amount of the complex as well; however, it affected both topoisomerase II and I, and its cytotoxicity was by far weaker than other antitumor agents tested. These observations indicate that the mode of action of genistein on the enzymes is different from that of those well studied topoisomerase inhibitors. It is necessary to test the effect of genistein on DNA strand scission, DNA intercalation, and so on.

Flavonoids have been known to have various pharmacological activities (13). For instance, the effect on carcinogenesis was also extensively studied using a flavonoid quercetin (14). It was reported that quercetin inhibited tyrosine-specific protein kinase (15). Akiyama et. al. have reported recently the inhibitory effect of genistein on tyrosine kinase (7).

Tyrosine kinases have been considered to play an important role in cell growth; the tyrosine kinase activity associated with $p60^{src}$ responsible for the transformation of fibroblasts has been reported to be inhibited by a serine protease inhibitor, TLCK (16). Since TLCK was ineffective in increasing topoisomerase-DNA complex in cells, it was used as a reference compound at the present study. As described in the Result section, TLCK did not inhibit the transformation caused by the oncogenic [Val 12]Ha-ras. Hence the inhibitory effect of genistein on the transformation of NIH 3T3 cells by transfection with pEJ plasmid appeared to be independent of the inhibition of tyrosine kinase. However, it remained unclear whether topoisomerase played a certain role in the transformation promoted by oncogenic Ha-ras; transformation experiment performed at the present study is composed of multi steps from the transfection with ras oncogene through the growth of transformed cells. The inhibitory effect of genistein in that experiment would result simply from the suppression of growth of the transformed cells.

Interesting observation was that genistein selectively suppressed the growth of the oncogenic [Val 12]Ha-ras-transformed cells at prolonged culture. Although the doubling time of the transformed (EJ) cells was much shorter than that of normal NIH 3T3 cells, this action of genistein seems not due to the cytotoxicity against rapidly dividing cells. In case of well known topoisomerase inhibitors and the other cytotoxic antitumor agents, they inhibit the growth of NIH 3T3 cells as well as tumor cells. The effect of genistein seems to resemble that of ditercalinium, which was reported to induce delayed toxicity in the cells sensitive to this agent (17). It was also shown that it inhibited the formation of covalent complex between topoisomerase II and DNA (18). As for TLCK, it has been reported that TLCK induced the phenotypic reversion of avian sarcoma virus-transformed cells to normal (19), and TLCK inhibited selectively the growth of SV40-transformed SV3T3 cells (20). Although cell lines used were different, our experimental result was reproducible and apparently the opposite of the previous report (20); TLCK inhibited the growth of the normal cells but not the transformed cells. These evidences suggest that the growth suppression by genistein was not caused by the common activity between genistein and TLCK. Moreover, at least one of the essential functions in NIH 3T3 cells is sensitive to TLCK. While in the cells transformed by the oncogenic ras, it was conceivable that such an essential function aquired resistance to TLCK, or other TLCK-insensitive ones.

In conclusion, genistein is a unique topoisomerase inhibitor effective in selectively suppressing the growth of cells transformed by the oncogenic ras. Genistein is surely a member of less toxic topoisomerase inhibitors, which may include ditercalinium. Because of the multifunction of genistein, it is difficult to elucidate its mechanisms of cell growth suppression so far,

however, this agent would be a useful tool for the research on the role of topoisomerase. Topoisomerase inhibitors of this type should be extensively studied since they may act on tumor cells more selectively than current anticancer drugs.

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