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INHIBITION BY FLAVONOIDS OF RNA SYNTHESIS IN PERMEABLE WI-38 CELLS AND OF TRANSCRIPTION BY RNA POLYMERASE II

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Abstract—The effects of various flavonoids on RNA synthesis in permeable human fibroblasts or on transcription with mouse RNA polymerase II were studied. Quercetin or kaempferol inhibited transcription in permeable cells but flavone did so only slightly. In the transcription of naked DNA with purified RNA polymerase II, mutagenic quercetin, kaempferol or fisetin strongly inhibited the reaction but non-mutagenic or weakly mutagenic flavone and chrysin inhibited it only weakly. Quercetin seems to inhibit the transcription by interaction with the enzyme.

Flavonoids are plant pigments and are widely distributed among foods [1, 2]. Genetic studies indicate that flavonoids induce forward mutation in bacteria [3–5] and in mammalian cells [6–8]. Flavonols increase the mutation frequency at thymidine kinase locus, but loci of hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase and Na^+/K^+ ATPase are affected only slightly in Chinese hamster ovary cells [8]. They also induce chromosomal aberrations, but sister chromatid exchange is marginally affected [8]. There are, however, conflicing results on the carcinogenicity of flavonoids. Umezawa *et al.* [9] reported that quercetin induces transformation of hamster embryonic cells *in vitro*, but transformation of Balb/c 3T3 cells is not induced significantly by quercetin [7]. Pamukcu *et al.* [10] found that quercetin is carcinogenic in rats, but several other investigators [11–13] reported that carcinogenicity of flavonoids is negative.

From the biochemical studies it has been reported that flavonoids have a wide range of actions in mammalian cells (for review, see Ref. 14). They inhibit Ca^{2+} influx [15], membrane ATPase [16], cAMP-dependent protein kinase [17] and incorporation of [^{3}H]thymidine, uridine or leucine into acid-insoluble fractions [18]. Quercetin is reported to increase cAMP level in ascites tumor cells [18]. Nishino *et al.* [19] found that quercetin inhibits the enhancement of phospholipid metabolism induced by tumor promotors.

We examined the effects of some flavonoids on cellular reactions involving DNA and found that they inhibited DNA and RNA synthesis in permeable cells, and that mutagenic flavonoids strongly inhibited RNA polymerase II activity.

MATERIALS AND METHODS

Chemicals. Quercetin (Tokyo Kasei Co.), kaempferol (Sigma Chemical Co.), fisetin (Tokyo Kasei Co.), flavone (Aldrich Chemical Co.) and chrysin (Aldrich Chemical Co.) were dissolved in dimethyl sulfoxide at 20 mg/ml as stock solutions.

Permeable cells and DNA or RNA synthesis. Normal human fibroblasts (WI-38) were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% newborn calf serum. Cells at population doublings of 30–40 were used. Cells were trypsinized and permeabilized with lysolecithin as described previously [20].

The permeable cells were incubated with [^{3}H]CTP in a transcription mixture as described before (5–8 × 10^4 cells/50 μl) [21], and the transcription with endogenous RNA polymerases was measured by counting radioactivity in acid-precipitable fractions. Specific activity; 1000 cpm = 0.26 pmole. Nucleic acids were isolated from incubated permeable cells by the phenol extraction method and were digested with RNase A or DNase I (200 μg/ml). Essentially all the radioactivity was rendered acid-soluble by RNase A but was totally resistant to DNase.

DNA synthesis in permeable cells was measured with randomly growing cells following the method of Miller *et al.* [22].

Transcription with RNA polymerase II. RNA polymerase II was purified from mouse Ehrlich ascites tumor cells following the method of Bitter and Roeder [23] as modified by Sekimizu *et al.* [24]. The steps included solubilization with ammonium sulfate, DEAE-cellulose, phosphocellulose, DEAE-Sephadex, glycerol gradient centrifugation and DEAE-Sephadex. The final preparation of the enzyme gave a single band on non-denaturing gel electrophoresis, and eleven bands on polyacrylamide gels containing sodium dodecyl sulfate. Transcription was carried out with [^{3}H]CTP and WI-38 DNA as a template in a reaction mixture described previously [25].

RESULTS

Inhibition of nucleic acids synthesis in permeable cells. Graziani and Chayoth [18] reported that quercetin inhibits the incorporation of [^{3}H]thymidine,

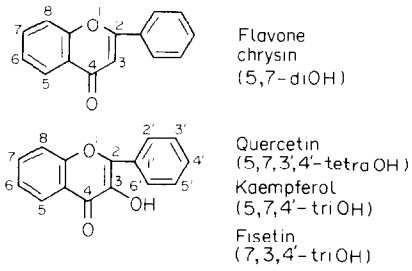

Fig. 1. Chemical structures of some flavonoides.

Table 1. Inhibition by flavonoids of DNA synthesis in permeable cells*

Addition	Concn (μg/ml)	[^{3}H]dCTP/10^6 (pmoles/10^6 cells)	%
Flavone	0	0.22	100
	50	0.22	91
	100	0.17	77
Chrysin	50	0.18	82
	100	0.14	64
Quercetin	50	0.15	68
	100	0.11	50
Kaempferol	50	0.11	50
	100	0.09	41
Fisetin	50	0.17	77
	100	0.16	73

* Permeable cells (8×10^4/50 μl) were incubated with [^{3}H]dCTP in the incubation mixture as described in the text at 37° for 15 min. Acid-insoluble radioactivity was measured.

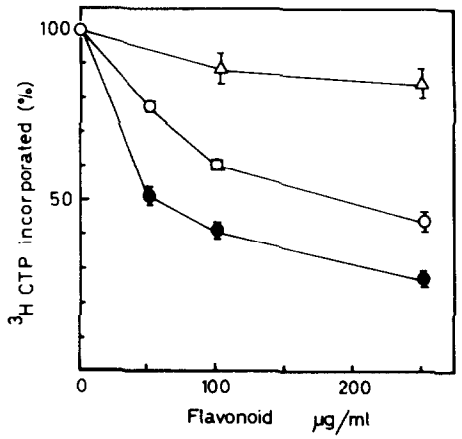

Fig. 3. Inhibition of RNA synthesis by flavonoids in permeable cells. Permeable cells were incubated as described in Fig. 2 in the presence of various concentrations of flavonoids for 20 min. Key: flavone (△———△), quercetin (○———○), and kaempferol (●———●).

uridine or leucine into acid-insoluble fractions in Ehrlich ascites cells, and we confirmed their results with WI-38 cells (data not shown). This rather non-specific inhibition of the macromolecular synthesis seems to be caused, at least in part, by a change in permeability barrier [16]. To study the mechanism for the inhibition more precisely, we examined the effects of flavonoids on DNA and RNA synthesis in permeable cells. Cells treated with weak detergents become permeable to nucleotides and incorporate [^{3}H]dCTP or CTP using endogenous polymerases. In these cells, membrane barrier is lost and the effects of drugs on membrane transport will become negligible.

The chemical structures of the flavonoids used in the present study are shown in Fig. 1. First, we examined the effects of flavonoids on DNA synthesis in permeable cells. The results in Table 1 indicate

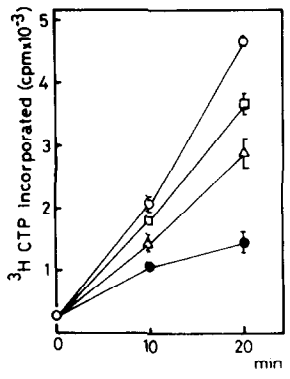

Fig. 2. Dose-dependent inhibition by quercetin of RNA synthesis in permeable cells. Permeable WI-38 cells were incubated with [^{3}H]CTP at 25° in the absence (○———○) or presence of quercetin, 50 μg/ml (□———□), 100 μg/ml (△———△) or 200 μg/ml (●———●). Acid-insoluble radioactivity was measured.

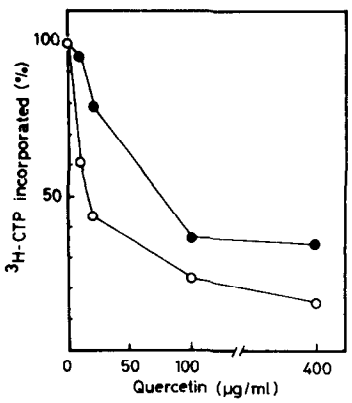

Fig. 4. Effect on quercetin on α-amanitin-sensitive and -resistant transcription in permeable cells. Permeable cells were transcribed in the presence (●———●) or absence (○———○) of 4 μg/ml α-amanitin. Various doses of quercetin were added at 0 min.

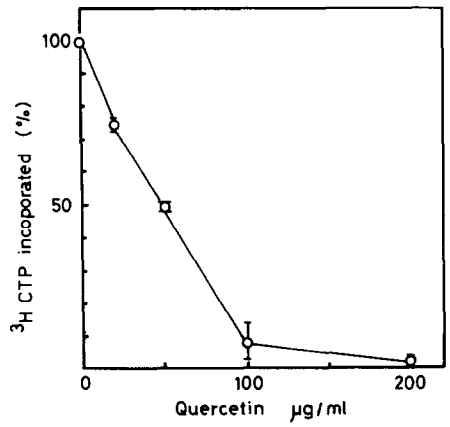

Fig. 5. Inhibition by quercetin of transcription with purified RNA polymerase II. DNA was transcribed with purified RNA polymerase II in the presence of various concentrations of quercetin at 25°. Reaction was stopped at 20 min and radioactivity was measured. The reaction mixture contained 0.4 μg DNA and 20 units of polymerase in 50 μl.

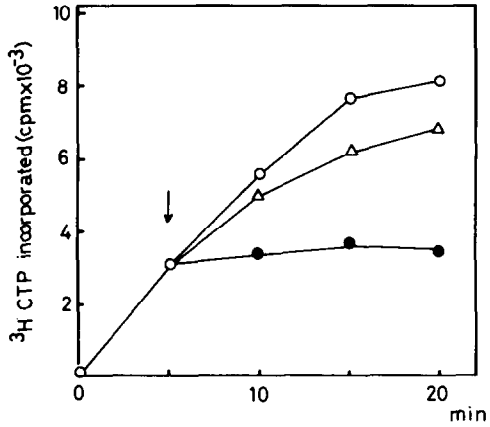

Fig. 6. Time course of inhibition of transcription. DNA was transcribed with RNA polymerase II at 25° as described in Fig. 5 and, at 5 min after the start of the reaction, quercetin (●——●) or rifampicin AF-013 (△——△) was added at 100 μg/ml. No addition is indicated by (○——○).

that all of the flavonoids tested inhibited DNA synthesis. Esumi *et al.* examined the effect of quercetin on purified *Escherichia coli* DNA polymerase I and found that there was no inhibition of DNA synthesis when native calf thymus DNA was used as a template (personal communication).

Figure 2 shows the effect of quercetin on RNA synthesis in permeable cells. It is evident that quercetin inhibited the incorporation of [^{3}H]CTP. Dose–response curves with non-mutagenic flavone and mutagenic quercetin or kaempferol are shown in Fig. 3. Quercetin and kaempferol inhibited the transcription strongly, but flavone inhibited it only slightly. Quercetin and kaempferol are reported to be strong mutagens and flavone to be weakly mutagenic, in the Ames' test, in the presence of an S9 mixture (rat liver microsomal fraction) [4, 14].

In permeable cells, the incorporation of [^{3}H]CTP reflects the sum of the activities of RNA polymerases I, II and III. To define which polymerase was inhibited by quercetin, the transcription reaction was carried out in the presence or absence of α-amanitin at a low concentration (Fig. 4). The drug at a concentration of 4 μg/ml inhibited [^{3}H]CTP incorporation by about 85%. The remaining activity can be regarded to represent the activity of RNA polymerases I and III. Quercetin inhibited both α-amanitin-sensitive and -resistant activities, but α-amanitin-sensitive RNA synthesis seems to be inhibited more strongly.

Effects of flavonoids on transcription with purified RNA polymerase II. In isolated nuclei, the transcription by endogenous RNA polymerases rep-

Table 2. Inhibition by flavonoids of transcription with RNA polymerase II*

Addition	Conc (μg/ml)	[^{3}H]CTP (pmoles/10^5 cells)	%
Flavone	0	2.386	100
	20	1.801	75.5
	100	1.078	45.2
Chrysin	20	2.062	86.4
	100	2.052	86.0
Quercetin	20	0.093	3.0
	100	0.006	0.2
Kaempferol	20	0.307	12.9
	100	0.006	0.3
Fisetin	20	0.367	15.4
	100	0.005	0.02

* Flavonoids were added in the reaction mixture containing 0.5 μg of DNA and 4 units of RNA polymerase II in 50 μl. The mixtures were incubated at 25° for 20 min. The values are the means of duplicate samples.

Table 3. RNA polymerase II assay using quercetin-treated and dialyzed polymerase or DNA*

	Treatment	Counts/min incorporated
DNA	−	2380 ± 30
	+	2570 ± 70
Polymerase	−	8360 ± 40
	+	3400 ± 180

* DNA (140 μg/ml) or RNA polymerase II (20 units/μl) was incubated with 200 μg/ml of quercetin for 5 min at 0°, and then dialyzed overnight against 50 mM Tris, pH 7.9, 5 mM $MgCl_2$, 1 mM ethylenediamine tetracetic acid, 25% glycerol and 1 mM dithiothreitol. Dialyzed DNA or polymerase was assayed as described in the legend for Fig. 5. The reaction mixture contained 2.8 μg/ml of dialyzed DNA and 5 units of polymerase, or 2.8 μg/ml of DNA and 20 units of dialyzed polymerase.

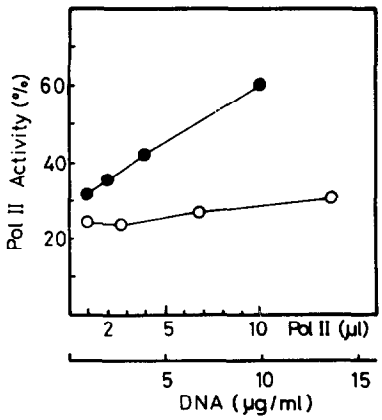

Fig. 7. Effect of the amount of DNA or RNA polymerase II on the inhibition by quercetin. Transcription was carried out in mixtures containing 10 units/100 μl of polymerase and various concentrations of DNA (○——○), or in the mixture containing 1.4 μg/ml of DNA and various amounts of polymerase (10 units/μl) (●——●). Activity was measured in the absence or presence of quercetin (10 μg/ml) with each concentration of DNA or polymerase II after incubation at 25° for 20 min.

resents only chain elongation, and initiation of the transcription was not observed [26]. Thus, it is possible that the transcription in permeable cells also manifests a chain elongation step. To study the mode of inhibition of RNA synthesis by flavonoids, effects on *in vitro* transcription with mouse RNA polymerase II were examined. RNA polymerase II was purified to homogeneity from mouse ascites tumor cells. Figure 5 shows that quercetin strongly inhibited the transcription of naked DNA template by RNA polymerase II. The effects of various flavonoids on the transcription were studied using non- or weakly mutagenic flavone and chrysin, and with the strong mutagens quercetin, kaempferol and fisetin [4, 14]. From the results shown in Table 2, it is clear that quercetin, kaempferol and fisetin inhibited the transcription strongly, whereas flavone and chrysin inhibited it only weakly.

When quercetin was added at 5 min after the start of the transcription reaction, the transcription was arrested promptly, in contrast to the case of rifampicin which is known to inhibit the initiation of transcription (Fig. 6). These results suggest that quercetin inhibited at the elongation step of the transcription.

We next questioned whether the inhibition of RNA polymerase II activity by quercetin was due to the effect on the enzyme or the template. First, DNA or RNA polymerase II was incubated with quercetin (200 μg/ml) for 5 min at 0° and then was dialyzed extensively. Dialyzed polymerase or DNA was added to the reaction mixture. The results of Table 3 indicate that quercetin had no effect on DNA after dialysis, but RNA polymerase II activity was inhibited by about 60%. Next, doses of DNA or polymerase II were increased in the reaction mixture in the presence of 0 or 10 μg/ml of quercetin which inhibits the reaction partially (Fig. 7). The inhibitory effect was not affected when DNA concentrations were increased from 1.4 to 14.2 μg/ml. At higher concentrations of DNA, the rate of transcription was lowered. On the other hand, the percentage of inhibition of transcription decreased when the concentrations of RNA polymerase II were increased. From these results, we conclude that quercetin interacts with RNA polymerase II and inhibits the transcription.

DISCUSSION

Quercetin and related flavonoids have been known to inhibit various metabolic processes in mammalian cells. The present study demonstrates that quercetin, kaempferol and fisetin inhibited the transcription with RNA polymerase II, whereas flavone and chrysin affected the activity only weakly. Graziani and Chayoth suggested that the apparent inhibition of cellular DNA or RNA synthesis may be due to increased cAMP level [18], but the results shown in the present paper indicate that this inhibition is due, at least in part, to the inhibition of RNA polymerase activity. The inhibitory effect of quercetin was observed both in α-amanitin-sensitive and -resistant transcription in permeable cells. Flavonoids inhibited DNA synthesis in permeable cells as well, but the inhibition was observed both with non-mutagenic and mutagenic flavonoids (Table 1).

From the experiments using purified RNA polymerase II, we conclude that quercetin inhibits the transcription by inactivating the polymerase, although the precise mechanism remains to be elucidated.

Many papers have reported that flavonoids induce somatic mutations in mammalian cells [3, 6–8] as well as in bacteria [3–5]. The inhibition of transcription is not regarded to be the direct cause of somatic mutations, but it is interesting that strong mutagens inhibited the RNA polymerase II activity and non-mutagens did so only weakly.

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