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cAMP-independent Protein Kinase Activity is Correlated with Growth of Rat Mammary Tumors*

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Abstract—*An important portion of the protein kinase activity in the 7,12-dimethylbenz(a)anthracene (DMBA) induced rat mammary tumour is inhibited by the bioflavonoid quercetin* (10^{-4} M). *By partial purification on a DEAE Cellulose column it was shown that the quercetin-inhibitable enzyme activity can be eluted in a separate peak which contains markedly reduced cAMP-dependent protein kinase activity. Since quercetin does not affect the cAMP-dependent protein kinase activity, this drug becomes a potent tool for the quantitation of this special activity in the tumor. By hormonal manipulation, namely ovariectomy and estrogen treatment, it was shown that changes in the growth rate of the tumour were closely correlated with the magnitude of these special protein kinase activities. These results suggest a possible cause-and-effect relationship between cyclic AMP-independent protein kinase activity and tumour malignancy in this chemically induced tumor. These data are similar to recent findings in viral-induced malignant transformation.*

INTRODUCTION

THE DMBA-induced rat mammary tumor retains some of the hormone responsiveness of the normal mammary gland. For example, the growth pattern of the majority of these tumours is hormone-dependent, stimulated by estrogens [1] and prolactin [2] and inhibited by antiestrogens [3] or by hormonal treatment [1]. However, the production of some hormone-induced proteins such as caseins is attenuated [4], suggesting a different stage of differentiation of these tumors. On the other hand, malignant transformation may cause the expression of some specific proteins which are not expressed normally to the same extent.

Recently it was demonstrated that the protein which appears after malignant transformation by avian sarcoma virus [5] and leukemia virus [6] is associated with cyclic AMP (cAMP)-independent protein kinase activity. Even some chemically induced transformation such as occurs after the administration of DMBA in bladder epithelial cells induces such protein kinase activity [7].

Recently we reported [8] the presence of Ehrlich ascites tumor cells of cAMP-independent protein kinase activities which are inhibited by quercetin. This drug is a bioflavonoid which occurs in many fruits, vegetables and other foods. Its inhibitory effect on growth and proliferation of malignant cells *in vitro* was extensively investigated [9], but the reason for its preferential inhibitory effect on malignant cells is unclear. The possibility that the cAMP-independent protein kinase activities are also found in other tumors and are correlated to tumor proliferation are studied in this paper. The DMBA-induced rat mammary tumor is specially suitable to study these questions because this tumor can be easily manipulated *in vivo* by hormonal means.

We report in this communication that the protein kinase activity which is inhibited by quercetin is an important fraction of the total protein kinase activity of these tumors. Moreover, this activity is significantly lower in regressing

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tumors after ovariectomy compared to that found in growing tumors in intact animals or in estrogen-treated ovariectomized animals.

MATERIALS AND METHODS

DMBA-induced tumors

Fifty-day-old female Sprague–Dawley rats were given 20 mg DMBA in soya oil by gastric intubation. Palpable tumors, which appeared 1–3 months after induction, were measured by calipers under light ether anesthesia at 3-day intervals. After a growth period of 2–3 weeks tumor regression was caused by bilateral ovariectomy under light ether anesthesia. Regrowth of tumors was induced by subcutaneous injection of estradiol (5 μg/day in saline containing 5% ethanol). Animals were killed at different stages of the above treatment and the tumors were removed and kept frozen at -60°C.

Preparation of crude cytosols

Frozen tumors were weighed, thawed and homogenized by all-glass homogenizers in 5 vol. of buffer containing 0.25 M sucrose, 2mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl and 20 mM Tris–HCl at pH 7.5. The homogenate was centrifuged at 800 *g* for 10 min. The pellet was washed in the original volume of the same buffer. The wash was combined with the first supernatant and was centrifuged at 35,000 *g* for 40 min. The supernatant was used for protein kinase assay.

Partial purification of protein kinase

Protein kinases from tumor cytosols were purified essentially as described by Trough and Traut [10]. The procedure included $(NH_4)_2SO_4$ precipitation, dialysis and DEAE-cellulose chromatography.

Protein kinase assay

The protein kinase activity was determined as described previously [11]. The reaction mixture, at a final volume of 0.25 ml, contained: 50 mM potassium phosphate buffer (pH 7.5), 10 mM magnesium acetate, 1 mM theophylline, 0.6 mg histone or casein and 0.5 mM ATP together with 1–2 μCi of [^{32}P]-ATP. Cyclic AMP was used at 5 μM. Quercetin was dissolved in DMSO. The final concentration of solvent in the assay was 3%. The reaction was started with the enzymatic preparation (50–200 μg) and carried at 30°C for 10 min. The cAMP-dependent protein kinase was calculated by subtraction of the protein kinase activity in the absence of cAMP from the activity in its presence. Protein concentrations were determined by the method of Lowry *et al.* [12], with crystalline bovine serum albumin as standard.

Materials

[^{32}P]-ATP was obtained from Nuclear Research Center, Negev, Israel. Calf thymus histone IIA, α-casein from bovine milk, quercetin, cAMP, theophylline, DEAE-cellulose and DMBA were obtained from Sigma.

RESULTS

The effect of quercetin on protein kinase activities

Protein kinase activities in DMBA-induced tumor cytosols were measured with both histone and casein as the protein substrates. Histone was a better substrate for the cAMP-dependent protein kinase, which showed a 3–5 fold stimulation over the activity in the absence of the cyclic nucleotide. The cAMP-dependent stimulation was unaffected by quercetin up to 10^{-4} M (Fig. 1). The activity without cAMP using both substrates was inhibited by quercetin, but since this activity was much higher with casein than with histone, we used casein for all measurements of cAMP-independent protein kinase activity. The inhibition of this activity by quercetin reached 70% at 10^{-4} M (Fig. 1). Half of that maximal inhibition was achieved at 2×10^{-5} M quercetin.

We repeated the enzyme assays after partial purification of the cAMP-independent protein kinase (peak III from DEAE cellulose chromatography [8, 10]). The results with histone (Table 1) shows that most of the cAMP-dependent protein kinase had been removed from this fraction since less than 50% stimulation was achieved with

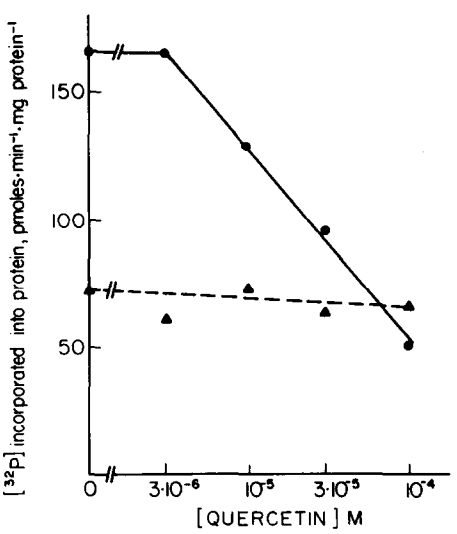

Fig. 1. Effect of quercetin on protein kinase activity in tumor cytosol. Protein kinase activity was measured in cytosol prepared from individual tumors, as described in the Materials and Methods section. ---- cAMP-dependent protein kinase activity was calculated from the difference in activity in the presence and absence of cAMP 5.10^{-6} M with histone as substrate. —— cAMP-independent protein kinase activity was measured with casein as a substrate.

Table 1. Effect of quercetin and protein kinase inhibitor on DEAE cellulose fractionated protein kinases

Additions	Protein kinase activity (pmol incorporated in protein/min/mg protein) Histone	Casein
None	340	1170
Quercetin (10^{-4} M)	140	440
PKI (100 μg protein)	340	1530
cAMP (5×10^{-6} M)	455	1380
cAMP + quercetin (10^{-4} M)	210	570
cAMP + PKI (100 μg protein)	410	1650

The $(NH_4)_2SO_4$ precipitate of tumor cytosol was chromatographed as described previously [8]; the peak fraction was used in the assay as representative of peak III. Activities were measured with histone or casein as indicated.

cAMP, in contrast to a 300–500% stimulation with the crude enzyme. The lack of inhibition of the cAMP-independent activity by the protein kinase inhibitor (PKI,100 μg/ml) shows that this activity does not represent the free catalytic subunit of the cAMP-dependent enzyme. The potency of our PKI preparation was validated in separate experiments. This preparation completely abolished the cAMP-stimulated protein kinase activity in tumor cytosol (results not shown).

In agreement with the results obtained with the crude cytosol, the cAMP-independent protein kinase activity in the purified fraction was higher with casein than with histone. The activity with both substrates was inhibited by quercetin (Table 1). These results allow us to quantitate a certain portion of the cAMP-independent protein kinase activities as that activity measured with casein and inhibited by the drug. We will refer to this activity subsequently as quercetin-inhibited protein kinase.

Correlation of tumor growth rate with protein kinase activity

In this study we included only tumours that responded to the hormonal treatment by significant regression after ovariectomy (phase II) and by regrowth during estradiol treatment (phase III). The average change in tumor surface area between different phases was approximately 3-fold (Fig. 2). Changes in size of the individual tumors varied between 2-and 10-fold.

Protein kinase activities were measured in the cytosols of tumors from all three growth phases (Table 2). The quercetin-inhibited protein kinase was more that 2-fold higher in the growing tumors than in the regressing ones. This activity was high in the growing tumors in the intact

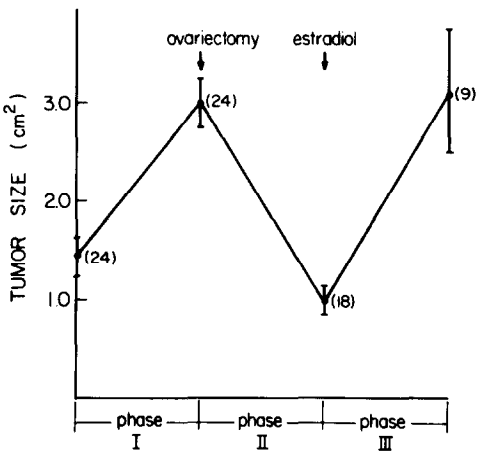

Fig. 2. Growth pattern of hormonally manipulated DMBA-induced mammary tumors. Tumor measurements were done at 2–3-day intervals. Only the measurement in the beginning and the end of each phase is given as mean ± S.E.M. (number of tumors in parentheses). Estradiol was administered s.c., 5 μg/day. The mean length of each phase was 14 days (8–20 days *in individual tumors).*

animals (phase I). It was significantly reduced ($P < 0.01$) after ovariectomy (phase II), parallel to tumor regression (Fig. 2). Moreover, when the tumors were induced to regrowth (phase III) this specific protein kinase activity was significantly increased ($P < 0.01$). No significant change was found in the cAMP-dependent protein kinase activities (Table 2) in cytosols prepared from tumors removed during the three growth phases ($0.1 < P < 0.2$).

DISCUSSION

The presence of cAMP-independent protein kinase activity, which is inhibited by quercetin in

Table 2. Quercetin-inhibited and cAMP-dependent protein kinase activities in growing and regressing tumor cytosols.

Phase	Protein kinase activity (pmol ^{32}P incorporated into protein/min/mg) Quercetin inhibited	cAMP-dependent
I Growing	82.3 ± 20.3(6)	133.8 ± 31.7 (6)
II Regressing after ovariectomy	28.4 ± 7.1(13)	110.0 ± 23.2 (13)
III Growing after estradiol administration	88.1 ± 12.8 (7)	75.6 ± 24.8 (7)

The quercetin-inhibited protein kinase activity was measured in the presence of casein as substrate and was calculated from the difference in activity in the absence and presence of the drug (10^{-4} M). The cAMP-dependent protein kinase activity was measured with histone as substrate and was calculated from the difference in activity in the presence and absence of the cyclic nucleotide 5.10^{-6} M. For full phase definition see Results section and Fig. 2. Mean ± S.E.M. (number of tumours in parentheses).

Erhlich ascites tumor cells but not in muscle tissue [8], stimulated us to study the possibility that this activity may be correlated to tumor proliferation, which is known to be inhibited by the drug [9]. Our findings support this idea since a specific class of protein kinase activity which is cAMP-independent and quercetin-inhibited is also abundant in the DMBA-induced mammary tumor and its activity is correlated with tumor growth. The decrease in quercetin-inhibited protein kinase activity in regressing tumors was reversed in regrowing tumors after hormone administration.

In contrast to the change noticed in cAMP-independent protein kinase activity in these tumors, no significant change was found in the cAMP-dependent protein kinase activity. Using similar tumors, Cho-Chung and her colleagues found an increase in cAMP-dependent protein kinase activity in cytosols of regressing tumors compared to growing ones [11]. This increase was evident only 6 hr after ovariectomy and disappeared 6 days later, which is in agreement with our results, which showed no significant change in tumors that were removed 8–20 days post-ovariectomy. After separating type II cAMP-dependent protein kinase, Cho-Chung *et al.* [13] found a regression-associated increase in this activity even 3 days after ovariectomy. Thus it is still possible that there is a persistent increase in a specific cAMP-dependent protein kinase of regressing tumors, but such an increase was not significantly evident in the unfractionated cytosols that we used in this study.

In contrast to the results of the cAMP-dependent protein kinase activity, a high degree of statistical significance was achieved in the results of the cAMP-independent activity. This is in spite of the small number of animals in each of the experimental phases, and is due to the relative uniformity of the results. The one exception was the phase I tumors, in which 2 out of 6 showed results which were in the range of phase II. These growing tumors cannot be identified as hormone-dependent with the same certainty as tumors of the second and third phases since hormone-dependency cannot be determined before hormonal manipulation, and hormonally independent tumors in a growth phase may be different.

The changes in tumor size, which are associated with the changes in the hormonal milieu in the animal, were shown in our previous studies to be directly correlated with estradiol and progesterone receptor levels in the tumor cytosol [1,3]. This by itself does not necessarily suggest a direct effect of estrogen or other hormones on the quercetin-inhibited protein kinase activity. More experiments are needed to confirm or deny this possibility.

The possibility that phosphorylation of specific cellular targets might account for transformation of the host cells by various protein kinases derived from viruses or produced by the host cell itself is now more than an intriguing hypothesis [14]. It is of special interest that quercetin was found to inhibit tyrosine phosphorylation by the cyclic-nucleotide-independent transforming protein kinase, pp 60^{src} [15]. These results support the possibility that the quercetin-inhibited protein kinase activity in DMBA-induced tumors described in this study is related to protein kinase activity affecting different transforming processes.

Moreover, phosphorylation of proteins was implicated not only in the mechanism of malignant transformation, but also in normal growth control. For example, epidermal growth factor (EGF) was found to affect tyrosine phosphorylation in its specific membrane receptor [16]. Recently it was reported [17] that platelet-derived growth factor stimulates similar phosphorylation of a membranous protein by specific phosphorylation, suggesting that such activities are important for the regulation of growth processes. EGF was reported to stimulate the growth of the MCF-7 human breast cancer cell line [18] and rodent mammary cancer *in vitro* [19].

In this study we report direct correlation of tumor growth with quercetin-inhibited phosphorylation activity in DMBA-induced mammary tumors. The possibility that this phosphorylation activity is similar to that found in growth regulation processes is under study.

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