POLYAMINES AND TUMOR CELLS: EFFECT OF TRANSFORMATION OF CHICK EMBRYO FIBROBLASTS BY ROUS SARCOMA VIRUS ON POLYAMINE LEVELS

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SUMMARY: The effect of transformation of chick embryo fibroblasts, by Rous sarcoma virus, on intracellular polyamine levels has been studied. A good correlation between spermidine and cellular protein content has been demonstrated. Upon changing the medium, a sharp increase in spermidine level was noticed both in normal and transformed cells. This increase was accompanied by enhanced protein synthesis. The intracellular concentrations of spermine and spermidine were very similar in normal and transformed cells. On the other hand, significant differences in putrescine levels were demonstrated: in normal cultures the intracellular concentration of putrescine reached a plateau approximately 6 days after seeding, whereas a continuous rise of the diamine in transformed cells was noticed. These differences, which were observed in cultured cells, may explain the known accumulation of polyamines during neoplastic growth.

Numerous recent studies indicated that the naturally occurring polyamines-spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$ and putrescine, $NH_2(CH_2)_4NH_2$ accumulate during neoplastic growth (1-3). It has also been demonstrated that polyamine-conjugates are excreted in the urine of cancer patients (4-6). Moreover, this increased excretion of polyamines is the basis for a proposed test for the early detection of cancer (4,5,7).

Until now, polyamine synthesis has been studied either in tumor-bearing animals (8) or in tissue cultures of cell lines derived from tumors (9). The interpretation of the results is complicated by the lack of appropriate controls in which identical experimental conditions prevail.

To test the correlation of polyamine synthesis and neoplastic growth, an experimental model has been devised. This model, which involves the use of oncogenic viruses, has definite advantages which stem from the rapidity of transformation and from the ability to perform kinetic experiments under well controlled conditions. It

will be shown that transformation of chick embryo fibroblasts by Rous sarcoma virus (RSV) alters intracellular polyamine levels.

METHODS:

The B_{77} and Schmidt Rupin (S.R.) strains of Rous sarcoma virus (RSV) obtained respectively from the Departments of Virology and Developmental Biology of this Medical School, were stored at -70°C. Primary cultures of fibroblasts prepared from 11 to 13-day old chick embryos, were seeded on gelatin (0.1%)-coated 50mm plastic plates (Nunc) at a concentration of 2.5 to $3x10^6$ cells/plate. The cells were grown at 37°C in Eagle's minimal essential medium (Gibco), supplemented with 20% tryptose phosphate broth (Difco Lab.) and 5% inactivated calf serum. After 24 hours, the growth medium was removed and 0.2ml of either fresh medium or RSV (10^5 to 10^6 focus forming units/ml) was added to each plate. Incubation at 37°C was continued for another 45 min., followed by the addition of fresh medium. Transformation became apparent after 2-3 days.

For chemical analyses, the growth medium was removed and the cell layer washed twice with cold saline. The cells, collected by scraping with a rubber policeman, were precipitated with 3% (v/v) perchloric acid and centrifuged (200g, 10 min.). The resuspended pellet was dissolved overnight at 37°C in 0.1N or 1N NaOH and assayed for proteins (10). The $HClO_4$ extract was analyzed for polyamines by the method of Seiler and Wiechmann (11) as follows: to 0.2ml of the $HClO_4$ extract, 18.5mg of Na_2CO_3 and 0.4ml of dansyl chloride (30 mg/ml in acetone, Fluka Chemical Co.) were added. After storing overnight in the dark, excess dansyl chloride was converted to dansyl proline by the addition of 0.1ml of an aqueous proline solution (10mg). Thirty minutes later, the dansylated polyamines were extracted into 0.5ml benzene and then separated by thin-layer chromatography on silica gel G plates (300μm thick). The plates were developed twice in ethylacetate-cyclohexane (2:3) and scanned with a Turner model 111 Fluorometer. The area under each peak was then measured.

RESULTS:

Changes in protein levels during the growth of normal chick embryo fibroblasts are illustrated in Figure 1b. Similar changes were observed after infecting the cultures with either RSV-B_{77} or RSV-S.R (Figure 1b). In all of these cultures, protein levels increased precipitously and reached a concentration of approximately 6 mg/plate after 9 days. A study of cellular polyamine content showed (Figure 1a) that in normal chick

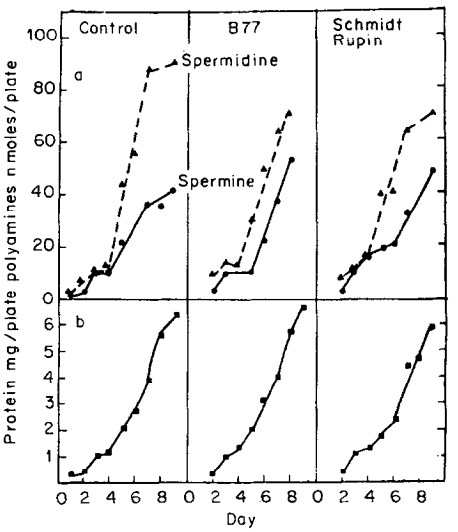

Figure 1. Changes in proteins and polyamines during the growth of normal and transformed chick embryo fibroblasts. Chick embryo fibroblasts (2.5 to 3.0x10^6 cells) were seeded on 50mm plastic plates and grown in Eagle's minimal essential medium. After 24 hr., growth medium was removed and 0.2ml of either fresh medium or Rous sarcoma virus (10^5 to 10^6 focus forming units/ml, strain B_{77} or Schmidt Rupin) was added to each plate. Medium was next changed on the fourth day and twice daily during subsequent incubation. Samples were assayed each day for proteins ■ —— ■ ; spermidine ▲ --- ▲ and spermine ● —— ●.
(a) Polyamines, (b) Proteins.

embryo fibroblasts spermidine increased moderately soon after seeding and then reached a plateau. Spermidine then rose sharply after the fourth day when the medium was changed. Subsequent changes of media resulted in similar changes in cellular spermidine (Figure 1a). Spermine also accumulated during the growth of the chick embryo fibroblasts in tissue cultures. Again, a plateau was observed during the second and third

day when no fresh medium was added. Changing the medium on the fourth day caused a significant increase in cellular spermine (Figure 1a).

Chick embryo fibroblasts, transformed by either RSV-B_{77} or RSV-S.R., also accumulated spermidine, in analogy to non-infected cultures. A slight depression was, however, noted during the last stages of growth (Figure 1a). Transformed chick embryo fibroblasts contained spermine in levels which paralleled those of the non-infected cultures (Figure 1a). It should, however, be noted that in normal and transformed cells the rate of spermine accumulation was slower than that of spermidine so that the molar spermidine/spermine ratio was usually greater than 1.0. When fresh medium was added to the cultures, and the proliferation rate increased, the molar spermidine/spermine ratio rose significantly. Table I shows that the initial high spermidine/spermine ratio declined during the third and the fourth day when either normal or transformed cells were analyzed. Changing the medium on the fourth day led to an increase in spermidine/spermine ratio (Table 1).

While transformation hardly affected the intracellular levels of spermidine and spermine , cellular putrescine content was markedly altered. It may be seen (Figure 2) that in the normal cultures a small increase in putrescine was noticed on the first day after seeding and a second rise was apparent on the fifth day, after changing the

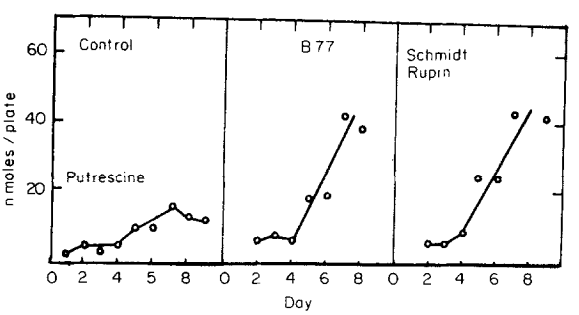

Figure 2. Changes in putrescine levels during the growth of normal and transformed chick embryo fibroblasts. Experimental conditions were as those described in Figure 1 except that samples were assayed for putrescine.

medium. Thereafter, the intracellular putrescine content remained fairly constant. When cells were analyzed after exposure to RSV, a significant increase in intracellular putrescine was noticed even before the appearance of foci (day 2 and 3 - Figure 2). Accumulation of putrescine continued throughout the incubation, with a sharp increase after changing the medium on the fourth day. It is evident that when most of the cells were transformed (after 6 to 9 days), intracellular putrescine concentrations were 3 to 5 times higher than in the non-infected culture.

DISCUSSION:

Data presented in this communication confirm previous findings (12) that correlate cellular polyamine levels with cellular proliferation rates (12). The high spermidine/spermine ratios depicted in Table 1 are typical for growing systems (13) and are in accord with published data (14). It is of interest that one day after

TABLE I Polyamines in normal and transformed chick embryo cells grown in vitro

Day of culture	Medium changed	Molar ratio spermidine/spermine		
		Normal cells	Cells transformed by $RSV\text{-}B_{77}$	RSV-S.R.
1	Infected	2.0		
2		2.7	3.0	3.1
3		1.0	1.4	1.2
4	+	1.0	0.9	1.0
5	+	2.2	2.8	2.3
6	+	3.0	2.4	2.1
7	+	2.2	1.6	2.2
8	+	1.7	1.2	1.8
9		2.0	1.5	1.2

changing the medium (on the fourth day) protein concentrations doubled (Figure 1b), while intracelullar spermidine increased by a factor of 3 to 5 (Figure 1a).

Putrescine also accumulated after changing the media of normal and transformed cultures (Figure 2). This is consistent with the finding that changing the media of cultured hepatoma (15) or baby hamster cells (16), results in the activation of ornithine decarboxylase. Ornithine decarboxylase, which catalyzes the synthesis of putrescine, is also elevated in various tumor cells (13) - this may explain the increase in putrescine content after transformation (Figure 2).

Results of experiments to be published elsewhere, indicated that intracellular putrescine remains elevated in RSV-transformed cells even upon subculturing. It therefore appears that the accumulation of polyamines is an intrinsic property of the malignant cell. The advantage of our system resides in the possibility of changing a normal cell into a malignant one, at will, under defined conditions and also permits a comparative study of normal and neoplastic growth. Our results support the notion that normal and tumor cells differ in their polyamine content. This has been demonstrated with two different strains of RSV. It remains, however, to be shown that changes in polyamine biosynthesis and accumulation is a general property common to all virus-transformed cells, and that similar changes cannot be induced by non-oncogenic viruses.

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