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Review article ■

Polyamines and Carcinogenesis

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SUMMARY

The naturally occurring polyamines, spermine, spermidine and the diamine putrescine are widespread in nature. They have been implicated in growth and differentiation processes. In 1967, we reported that cancer cells are rich in polyamines. Subsequently, it has been shown that polyamines are released from cancer cells and may be detected in body fluids such as urine, blood and cerebrospinal fluids. It has also been demonstrated that the increase in cellular polyamine levels is an early and an obligatory event in the process of malignant transformation. This increase in cellular polyamine concentration is due to the activation of ornithine decarboxylase (ODC), which catalyses the rate limiting step in polyamine synthesis by converting ornithine to putrescine. Assays of urinary and blood polyamines have been used to detect cancer and to determine the success of therapy. A sensitive, rapid, chemiluminescence-based method for the determination of diamines and polyamines was developed and 2.000 urine samples were tested. An interesting "gene therapy" system for injecting amine oxidases into normal and transformed cells was developed as follows: serum amine oxidase and porcine kidney damine oxidase were trapped within reconstituted Sendai virus envelopes. Chick or rat fibroblasts, transformed by Rous sarcoma virus, were more susceptible to the injected enzymes, compared to the normal culture, when macromolecular synthesis was tested. An *in vitro* chemosensitivity assay for the testing of the sensitivity of cancer cells from individual patients ("tailored treatment") was also developed. All these studies stress the importance of polyamines in carcinogenesis.

Key words: **polyamines, transformation, carcinogenesis, tumor viruses, urine analyses**

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INTRODUCTION

Naturally occurring polyamines are ubiquitous organic compounds (Figure 1), found in all eukaryotic and prokaryotic cells studied so far (1-6).

Polyamines have attracted considerable attention and during the past 20 years over 45,000 scientific papers have been written on this subject (7). Ornithine decarboxylase (ODC), which catalyzes the rate-limiting step in polyamine biosynthesis, has a short half-life (8, 9). Generally, polyamine biosynthesis is enhanced in proliferating systems such as embryonic tissues and in regenerating liver (4, 10).

Likewise, ODC may be regarded as a marker of proliferation, its activity is high during growth and declines when growth is arrested (11). Indeed, inhibiting polyamine biosynthesis by blocking ODC can be used for arresting the growth of parasites and for the chemoprevention of cancer (12). As cancer cells multiply rapidly, it has been expected that these cells will also be rich in polyamines.

Are polyamine levels higher in cancer cells?

In the course of investigations dealing with the influence of local biochemical conditions on the fate of tubercle bacilli *in vivo*, it was demonstrated that a powerful antimycobacterial action was exerted by the naturally occurring base spermine (13). Subsequently, it has been shown that spermine and spermidine inhibit bacterial growth, only if another tissue substance was present in the culture medium. Bovine serum amine oxidase was the activating factor (14). The purification of diamine oxidase, which catalyzes the oxidation of the diamine putrescine, was improved by Mondovi et al. (15, 16). The oxidation products of polyamines are highly toxic and they have been identified as aminoaldehydes and hydrogen peroxide (17). As these oxidation products inhibit the growth of bacteria (18, 19), we speculated that they would also interfere with the growth of cancer cells, which were known to proliferate rapidly. Ehrlich ascites cells were treated with purified serum amine oxidase in the presence of catalase (to rule out any effect exerted by hydrogen peroxide). These treated cells were then injected into mice and after 7 days tumor cells were collected from peritoneal cavities and their packed volumes were determined. A significant decrease in tumor cells was observed when amine oxidase was incubated with various amines. A control experiment, in which Ehrlich ascites cells were incubated with serum amine oxidases, gave surprising results: the growth of the treated cells in mice was also reduced by 20-50% even in the absence of added polyamines (20). This led to the hypothesis that Ehrlich ascites cancer cells contain polyamine which can be oxidized to toxic product by serum amine oxidase. This hypothesis was confirmed experimentally and substantial amounts of polyamines were extracted from Ehrich ascites cells and identified (21). This was the first evidence that cancer cells are rich in polyamines.

Is the increase in polyamine levels an early and an obligatory event?

The correlation between polyamine synthesis and neoplastic growth has been studied in tumor-bearing animals or in tissue cultures of cells derived from tumors. Such experimental models lack appropriate controls. In animals, tumor cells generally have a higher metabolic rate than normal tissues and *in vitro,* tumor cell lines differ physiologically from normal primary cultures. Thus, these experiments do not provide conclusive evidence as to the specific role which polyamines play in neoplastic growth. In an attempt to clarify this point, cultured cells were transformed by a temperature-sensitive tumor virus. In such a system, cancer can be induced at will and under optimal growth conditions, normal cells propagate at practically the same rate as transformed cells. Chick embryo fibroblasts were infected with wild-type or temperature-sensitive mutants of Rous sarcoma viruses at permissive and nonpermisive temperatures (22). Infection of the chick embryo fibroblasts with a wild-type Rous sarcoma virus resulted in a twentyfold increase in ODC activity. When cells were infected with the temperature sensitive mutant of the oncogenic virus, the activity of ODC was stimulated at the permissive (37^0) but not at the nonpermissive temperature (42^0). The activity of ODC increased within two hours when the temperature of the infected cells was shifted from 42^0 to 37^0 (Figure 2).

Shifting of temperatures of incubation caused comparable alterations in cellular putrescine levels. These studies demonstrated that those changes in cellular putrescine levels were early and obligatory events in the transformation process (23).

Another viral system, by which malignant transformation can be induced at will, was based on the use of a construct in which the oncogene Ha-*ras* was under the transcriptional control of mouse mammary tumor virus long terminal repeat promoter. In the presence of dexamethasone (Dex), the oncogene *ras* was expressed (Figure 3). The expression of *ras*, led to the activation of ODC and to the accumulation of polyamines in the transformed cells.

As expected, activation of c-Ha-*ras* by Dex and the accumulation of polyamines resulted in morphological changes (Figure 4).

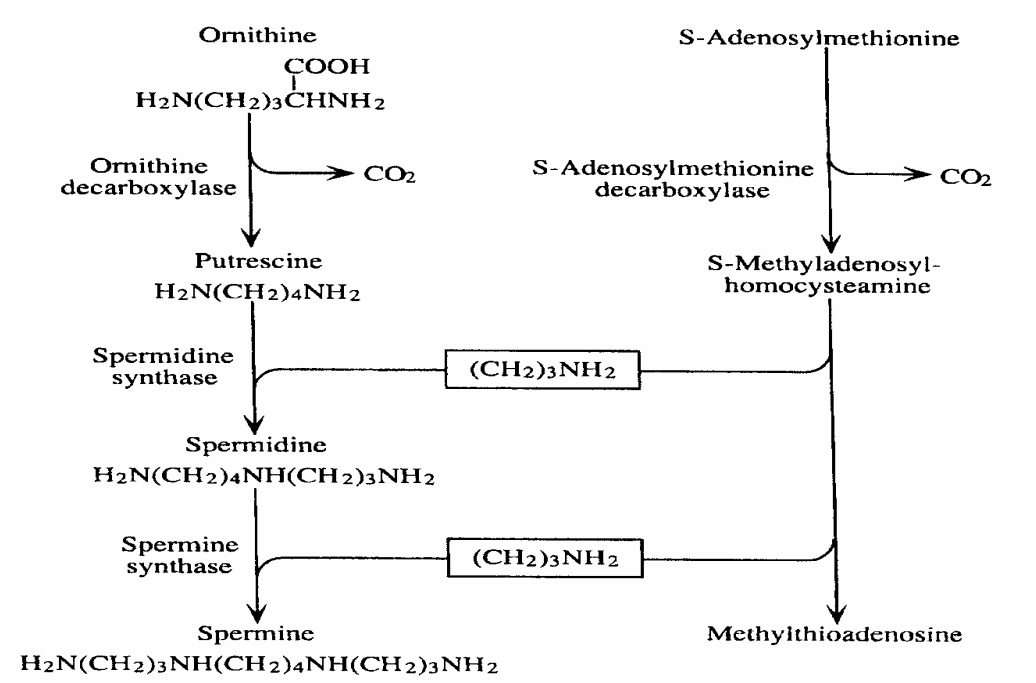

Figure 1. *Biosynthesis of polyamines*

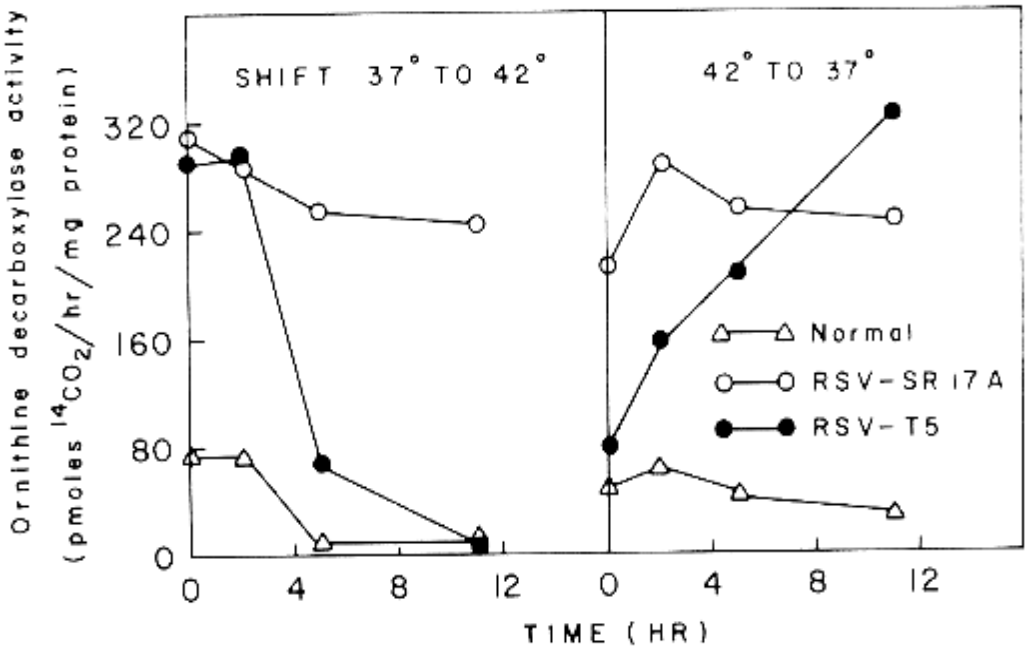

Figure 2*. Effect of temperature shift on ornithine decarboxylase activity in uninfected and infected chick embryo fibroblasts. RSV-SR174- wild type Rous sarcoma Virus; RSV-T5- temperature sensitive mutant. Cells were grown at 37^0 or 42^0 and after 7 days of incubation, temperature was shifted to the alternative one*

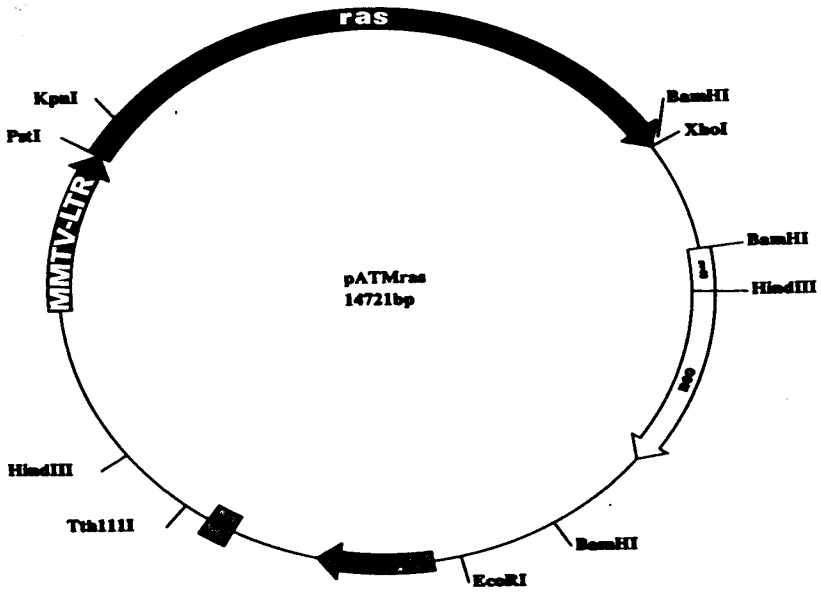

Figure 3. *Construct used to detect transformation NIH 3T3 fibroblasts, transfected with a construct, in which c-Ha-ras is under the transcriptional control of mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter*

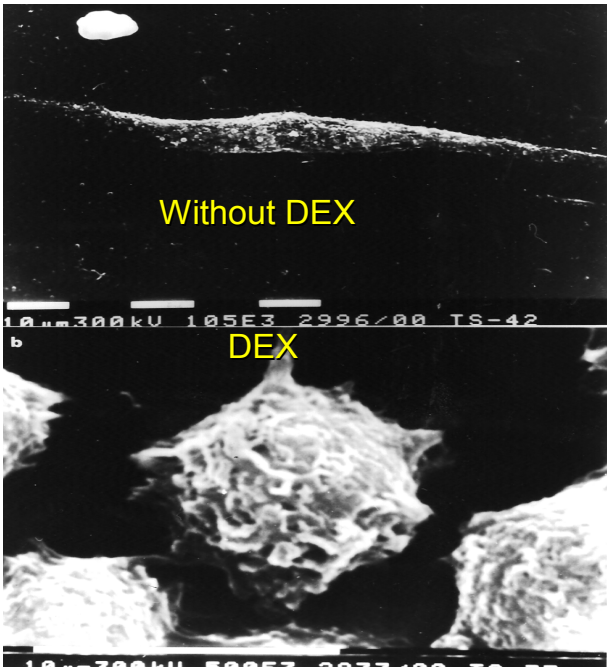

Figure 4. *Effect of dexamethasone on the morphology of MMTV - infected cells*

Analytical tools and clinical applications

Based on the report by Russell (24), numerous attempts were made to detect urinary polyamines for cancer diagnosis and therapy (25). It soon turned out that polyamines are excreted from tissues and cells as acetyl derivatives and therefore urine samples should be hydrolyzed, chemically or enzymatically, to yield free polyamines. The methods used to determine polyamines and diamines included: chromatographic methods (gas, ion-exchange and high-pressure chromatographic methods). The sensitivities and the specificity of the methods varied considerably (25). A specific method to determine polyamines and diamines was based on the use of specific enzymes. Polyamines can be oxidized by serum amine oxidase (15, 17) and putrescine by hog kidney diamine oxidase (16). Hydrogen peroxide which is the product of polyamine and diamine oxidation can be assayed by sensitive chemiluminescence methods, based on the emission of light in the presence of luminol (26).

The activity of polyamine oxidases can be inhibited by interfering compounds present in urine. To overcome this difficulty a "clean up" step was recommended before treating the samples with the enzymes. P-81 phosphocellulose paper strips were used for purification (26). The assay of urinary polyamines is illustrated in Figure 5. The first step was hydrolysis of urine samples by acid. Next, hydrolyzed sample were applied on phosphocellulose paper strips. After drying, paper strips were washed by dilute ammonium hydroxide solution to remove inhibitors which are not positively charged. Polyamines and diamines are then eluted from the paper by sodium chloride solutions. Eluates are then treated with specific oxidases and the amount of hydrogen peroxide formed is determined by chemilumscenceine (Figure 5).

Recovery of the patients as a result of therapy is reflected by the decrease in urinary polyamines which ranged from 30 to 20 nmoles per mg of creatinine (Figure 6). Similar values are observed when polyamines were assayed in the urine of normal individuals. The increase of urinary excreted compounds is related to the progress of the disease (Figure 7) and values are much higher than in the urine of normal controls. Urinary polyamine values are comparable to the results obtained by other cancer markers (Figure 7), such as C.E.A. (carcinoembryonic antigen) and CT (computed tomography).

NO EVIDENCE OF THE DISEASE

Follow-up studies can predict the recurrence of the disease even before the appearance of clinical symptoms. Urinary polyamine analyses also permit the evaluation of the stage of the tumor and can help in the early detection of cancer.

Cancer detection and therapy can also be based on the assay of polyamines extracted from erythrocytes of cancer patients (27).

In vitro chemosensitivity testing

Oncology is currently an empirical discipline in which all patients with a particular type of cancer are treated as though they were the same. Tumors of the same type show heterogeneity of chemosensitivity, and patients with apparently identical tumor histologies do not always respond identically to the same drug regimen. As in the treatment of infectious diseases, an *in vitro* chemosensitivity test for cancer could increase the chance of recovery, reduce undesired side effects and minimize the emerge of multi-drug resistance (MDR) variants. Therefore, a "tailored" cancer therapy, optimized to individual patients, is very desirable. An ideal *in vitro* chemosensitivity test should be based on a marker for proliferation. Such a marker should be universal, found in almost all cells, should have a short half-life, so that it would decay rapidly when cell proliferation is arrested. Ornithine decarboxylase meets these requirements and therefore can be used as a marker for proliferation to test the *in vitro* chemosensitivity of individual cancer patients (28).

The presence of ODC protein in individual cells was quantitatively detected by an immunofluorrescence assay, using an ACAS 570 computerized fluorescence microscope (29). It may be seen (Figure 8) that ODC protein, detected in the human epithelial carcinoma KB-3-1 cells, was grown in the absence of any drug. Vinblastie, which inhibits cell growth, caused the disappearance of ODC. On the other hand, ODC protein was detected in multidrug-resistant cells grown in the absence or presence of vinblastine. NIH 3T3 fibroblasts transformed by c-Ha-*ras* oncogene also contained ODC, which disappeared when cells were treated with cycloheximide (Figure 8).

A new method for the *in vitro* chemosensitivityy testing of human lymphoma and leukemia patients has been described (30). Ornithine decarboxylase - a marker for proliferation was detected in individual cancer cells by a quantitative immunohistochemical analysis using ODC antibody and a FITC-linked second antibody. A good correlation was found between the predicted chemosensitivity and the outcome of therapy. This approach of *in vitro* chemosensitivity testing opens new possibilities for the detection of multidrug resistant cells and could improve the treatment of cancer patients.

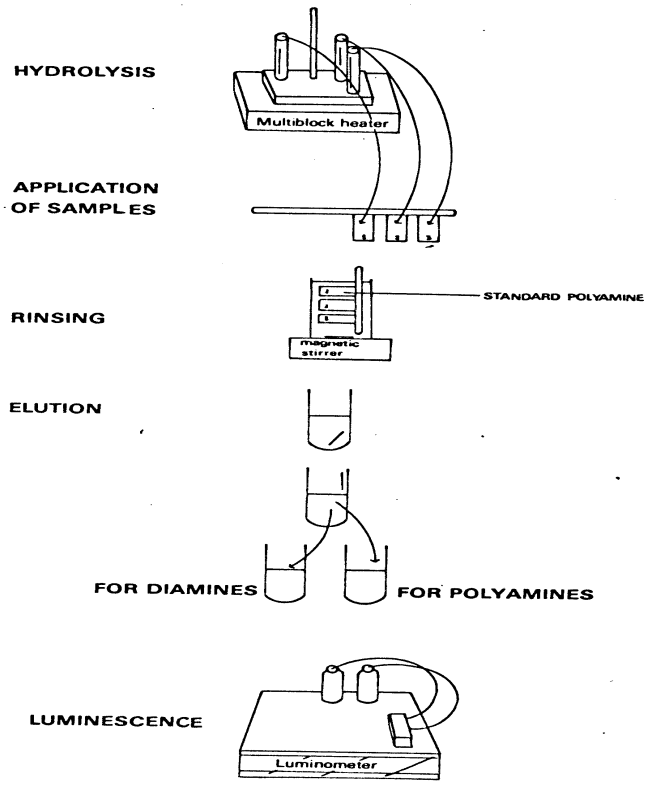

Figure 5. *Enzymatic assay of urinary polyamines and diamines*
Using this method, more than 2.000 urine samples were analyzed
It has been shown that 80-90% of cancer patients excrete polyamines and diamines in their urine

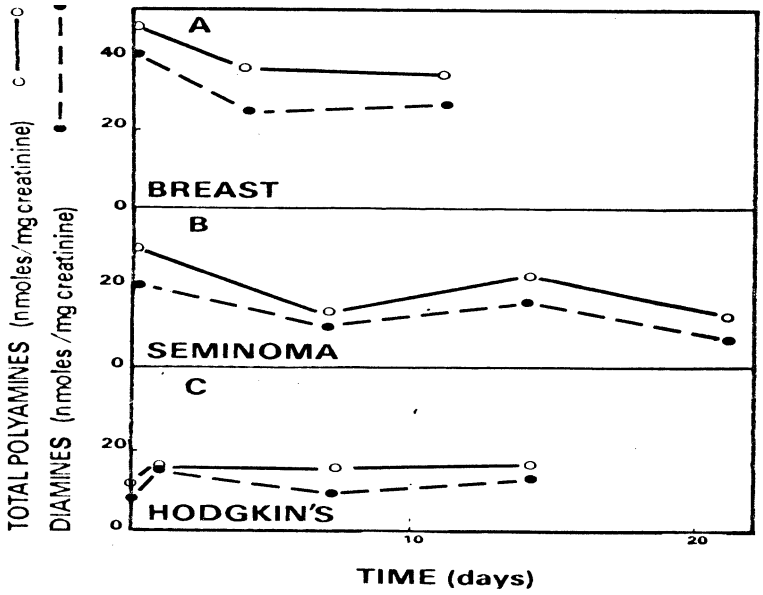

Figure 6. *Polyamines and diamines in the urine of patients who are recovering from the disease and are classified as N.E.D. (no evidence of the disease)*

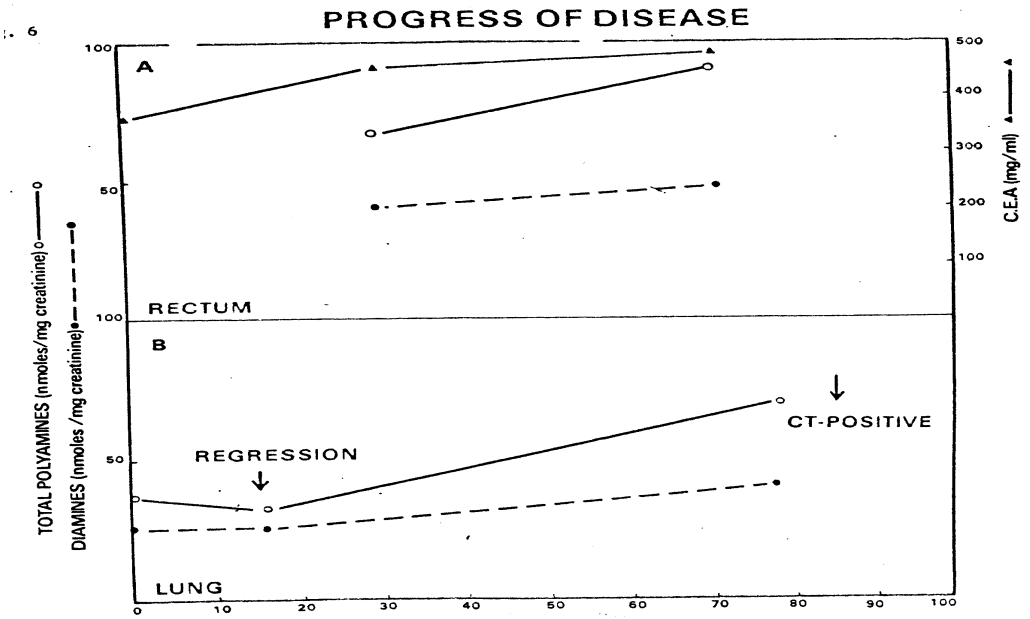

Figure 7. *Polyamines and diamines in the urine of cancer progressive disease patients*

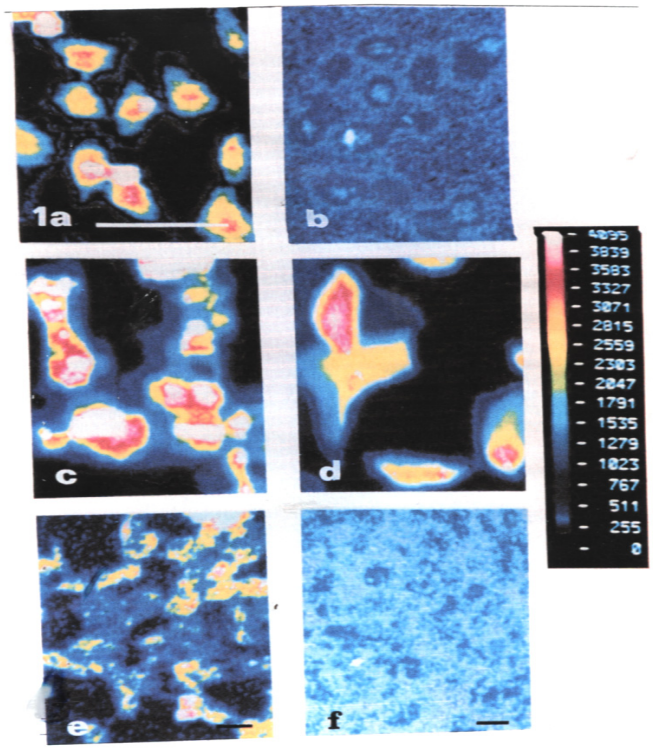

Figure 8. *Immunofluorescence image of ornithine decarboxylase in cultured cells.*
(a) *Wild-type of untreated human epithelial carcinoma KB-3-1 cells, grown for 48 h at 37^0*
(b) *KB-3-1 cells, treated with 0.5 mg/ml vinblastine for 24h*
(c) *Untreated human multidrug-resistant epithelial carcinoma KB-V-1 cells, grown for 48 h at 37^0*
(d) *KB-V-1 cells, treated with 0.5 mg/ml vinblastine for 24h*
(e) *Untreated c-Ha-ras-transformed NIH 3T3 fibroblasts*
(f) *c-Ha-ras-transformed NIH 3T3 fibroblasts, treated with 10 mg/ml cycloheximide for 3h*

Gene therapy - induced by polyamines

Cancer cells are rich in polyamines and these polycations can be oxidized by bovine serum amine oxidase to yield cytotoxic products. Based on these considerations, it is conceivable that cancer cells could be inactivated preferentially, as they contain polyamines which are the substrate of amine oxidases. Normal cells, which contain only small amounts of polyamines, should not be affected by the injected enzyme.

Sendai virus particles can be solubilized by detergents such as Triton X-100. Removal of the detergent leads to the formation of empty reconstituted virus envelopes. When soluble macromolecules are present in the detergent-solubilized fraction, they are trapped within resealed envelopes, reconstituted after the removal of the detergent.

Bovine serum amine oxides and hog kidney diamine oxidase were trapped within reconstituted Sendai envelopes and they retained their activity. It was predicted that cells rich in polyamines and diamines would be more susceptible to the injected oxidases, than others that contain polyamines and diamines at lower concentrations. When serum amine oxidase and/or diamine oxidase were microinjected into cultured fibroblasts of chick or rat embryos, a slight temporary inhibition in DNA and protein synthesis was observed. When these fibroblasts were transformed by Rous sarcoma virus, they were more susceptible to the injected enzymes than the normal cultures.

It may be seen (Figure 9) that normal chick embryos were not affected by the injected oxidases, unlike the transformed fibroblasts, in which macromolecular biosynthesis was significantly arrested (31).

Reconstituted virus envelopes were attached to the eukaryotic cells. No significant changes in the morphology of normal chick embryo fibroblasts were noted. On the other hand, chick embryo fibroblasts transformed by Rous sarcoma virus (Figure 10) were affected by the injected enzymes and holes were detected in the treated cancer cells (32).

Immobilized amine oxidase or immobilized diamine oxidase were injected into tumor-bearing animals (33, 34). A significant inhibition of the growth of the cancer cells was reported.

Averill-Bates et al. (35), immobilized bovine serum amine oxidase into poly(polyethylene glycol) particles. These were injected into mice carrying B16 tumor cells. Again, a remarkable decrease of tumor growth resulted.

It appears that immobilized amine oxidases have a potential therapeutic value. The inert liposomes used for trapping of the enzymes have an advantage by not being antigenic and do not trigger the production of immunological reactions. On the other hand, the reconstituted viral envelopes can be targeted and therefore may be more potent and specific.

It can be concluded that polyamines accumulate in cancer cells and that their identification in human body fluids can help to predict the course of cancer development and predict the relapse of the disease. Polyamines, or their biosynthetic enzymes can be used for a "tailored" individual chemosensitivity determination. Moreover, intracellular elevated polyamines in cancer cells can be used to preferentially inactivating those cells by injecting amine oxidases.

All these findings stress the importance of polyamines in understanding the process of carcinogenes, help cancer diagnosis and perhaps also provide new avenues for the treatment of cancer patients.

Culture	Incorporation (ct/min/culture dish)	
	Thymidine	Leucine
Normal cells	11,500	12,000
Normal cells+ microinjected oxidases	10,800	10,000
Transformed cells	10,125	10,000
Transformed cells+ microinjected oxidases	3,800	3,200

Figure 9. *Effect of microinjected amine and diamine oxidases on the synthesis of DNA and proteins by chick embryo fibroblasts*

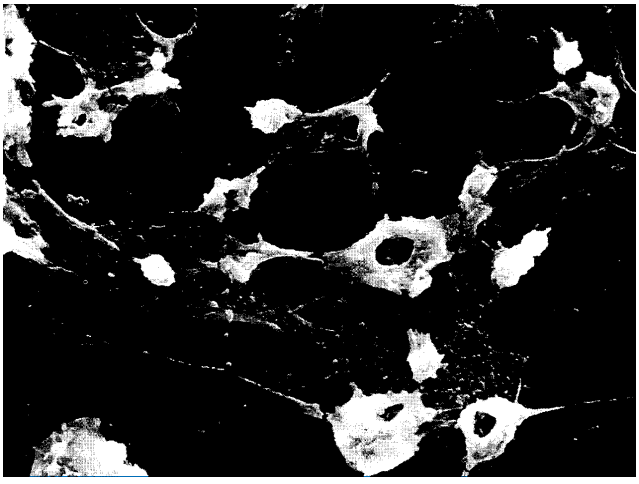

Figure 10*. Scanning electron micrograph of transformed chick embryo fibroblasts treated with reconstituted envelopes containing amine and diamine oxidases*

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POLIAMINI I KARCINOGENEZA

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Sažetak

Poliamini, spermin, spermidin i diamin putrescin su jako rasprostranjeni u prirodnom obliku i uključeni su u procese rasta i diferencijacije. Da su ćelije kancera bogate poliaminima opisali smo 1967. godine. Ubrzo je prikazano da ćelije kancera ispuštaju poliamine i da se oni mogu detektovati u telesnim tečnostima poput urina, krvi i cerebrospinalne tečnosti. Takođe je prikazano da je porast ćelijskog nivoa poliamina rani i obavezni događaj u procesu maligne transformacije. Ovaj porast u ćelijskoj koncentraciji poliamina dešava se zbog aktivacije ornitin dekarboksilaze (ODC), koja katališe nivo limitirajuće stope u sintezi poliamina konvertovanjem ornitina u putrescin. Testiranje poliamina iz urina i krvi je rađeno u cilju otkrivanja kancera i određivanja uspešnosti terapije. Razvijena je senzitivna i brza metoda hemiluminiscencije za određivanje diamina i poliamina i testirano je 2000 uzoraka urina. Interesantan sistem ''genetske terapije'' za ubacivanje amin oksidaze u normalne i transformisane ćelije razvijen je na sledeći način: serum amin oksidaze i diamin oksidaza dobijena iz svinjskog bubrega su zadržani u rekonstituisanim omotačima Sendai virusa. Fibroblasti embriona pileta ili pacova, izmenjeni virusom Rausovog sarcoma, bili su mnogo podložniji na ubačene enzime u poređenju sa normalnom podlogom, kada je testirana makromolekularna sinteza. Takođe je razvijen i *in vitro* test hemosenzitivnosti (''prilagođeni tretman'') ćelija kancera kod različitih bolesnika. Sve ove studije naglašavaju značaj poliamina u karcinogenezi.

Ključne reči: **poliamini, transformacija, karcinogeneza, tumorski virusi, analiza urina**