Biochemical Programs of Slowly and Rapidly Growing Human Colon Carcinoma Xenografts

George Weber,¹ Jean C. Hager,² May S. Lui, Noemi Prajda,³ Diana Y. Tzeng, Robert C. Jackson,⁴ Eiji Takeda,⁵ and John N. Eble

Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, Indiana 46223 [G. W., M. S. L., N. P., D. Y. T., R. C. J., E. T., J. N. E.], and Department of Medicine, Roger Williams General Hospital, Brown University School of Medicine, Providence, Rhode Island 02903 [J. C. H.]

ABSTRACT

The purpose of this investigation was to elucidate the enzymic programs of pyrimidine, carbohydrate, and purine metabolism and the pattern of pyrimidine and purine ribonucleotides in two lines of human colon carcinoma xenografts of different growth rates. The slower-growing colon tumor line was well differentiated; the more rapidly growing line was a poorly differentiated one. The carcinoma xenografts were carried in nude (athymic) mice.

The increased malignancy and growth rate of the rapidly growing colon tumor were characterized by a markedly amplified imbalance in the enzymic programs and nucleotide patterns. In the rapidly growing carcinoma line, the activities of the enzymes of the pyrimidine de novo biosynthesis (cytidine 5'-triphosphate synthetase, orotidine 5'-monophosphate decarboxylase, orotate phosphoribosyltransferase) and those of the salvage pathways (thymidine kinase, uracil phosphoribosyltransferase) were markedly higher than those in the slower-growing tumor line. The activities of the glycolytic enzymes (hexokinase, phosphofructokinase, pyruvate kinase) in the slower-growing tumor line were also elevated in the rapidly growing neoplasm. The activity of the enzyme that converts ribose 5-monophosphate into phosphoribosylpyrophosphate (phosphoribosylpyrophosphatase) was also augmented. In the purine metabolism of the rapidly growing carcinoma, there was increase in the activity of the first enzyme committed to de novo biosynthesis (glutamine phosphoribosylpyrophosphatase amidotransferase); by contrast, the activities of the opposing purine catabolic enzymes (xanthine oxidase, inosine phosphoribosyltransferase) were decreased. The activity of the enzyme that produces inosine 5'-phosphate from adenylates (adenosine 5'-phosphate deaminase) and of the rate-limiting enzyme of guanylate biosynthesis (inosine 5'-phosphate dehydrogenase) were elevated in the rapidly growing colon tumor lines.

The enzymes that were identified as progression linked in the rat hepatoma system were also progression linked in the two human colon carcinomas of different growth rates (cytidine 5'-triphosphate synthetase, thymidine kinase, hexokinase, phosphofructokinase, pyruvate kinase, inosine 5'-phosphate dehydrogenase, and adenosine 5'-phosphate deaminase).

There was a marked enlargement of the pools of adenylates, guanylates, uridylates, and cytidylates in the rapidly growing neoplasm. Particularly marked rises were observed in the concentrations of xanthosine 5'-phosphate, uridine 5'-diphosphate, cytidine 5'-phosphate, and cytidine 5'-diphosphate (11- to 18-fold).

The high activities of both de novo and salvage pathways of pyrimidine biosynthesis and the elevated enzymic capacities in carbohydrate catabolism and in purine biosynthesis, along with the large pools of pyrimidine and purine ribonucleotides, may account for, in part at least, the clinical difficulties encountered in the chemotherapy of human colon neoplasia.

The results indicate the applicability of the molecular correlation concept to human colon neoplasia and should be helpful in the rational design of enzyme pattern-targeted chemotherapy of colon tumors.

INTRODUCTION

In this laboratory, studies guided by the molecular correlation concept led to the discovery of an integrated pattern of enzymic imbalance that was linked with transformation and progression in a series of chemically induced, transplantable, solid rat hepatomas of different growth rates (13, 14). Recently, we also reported that the levels of certain purine and pyrimidine ribonucleotides and deoxyribonucleoside triphosphates in the hepatomas also were linked with transformation and progression (4, 5, 19).

The purpose of this investigation was to test the applicability of the molecular correlation concept to human colon tumor xenografts of different growth rates and differentiation. The studies also were planned to determine whether or not there are any enzymic activities or nucleotide concentrations that might serve as biochemical markers of growth rate and degrees of differentiation in the human colon tumors. It was also postulated that a deeper insight into the enzymic programs and nucleotide patterns might illuminate the biochemical pharmacological basis of the difficulties encountered in the drug treatment of human colon tumors.

The results showed that the approaches of the molecular correlation concept of neoplasia did apply to the human colon xenografts of different growth rates. The activities of certain key enzymes in pyrimidine, carbohydrate, and purine metabolism provided markers for the growth rate and degrees of malignancy of the human colon tumor xenografts. The increase in the pools of pyrimidine and purine ribonucleotides also provided a biochemical indicator of the degrees of malignancy.

¹ Recipient of USPHS Grants CA-13526 and CA-05034. To whom requests for reprints should be addressed.
² Present address: National Cancer Institute, Budapest, Hungary.
³ Recipient of USPHS Grant CA-18129.
The clinical difficulties in the treatment of colon tumors with single agents that inhibit solely the de novo synthesis (e.g., pyrazofurin, N-(phosphonacetyl)-L-aspartic acid) or utilize the salvage pathway for activation (e.g., 5-fluorouracil) might be accounted for, in part at least, by the presence of strong activities of the salvage enzymes and of the de novo synthetic enzymes of uridylate and cytidylate production. These observations should be helpful in the design of rational chemotherapy of human colon carcinoma.

MATERIALS AND METHODS

Nude (athymic) mice were inoculated s.c. with the tumor xenografts at 4 sites on the flanks. The mice were housed 5/cage in air-conditioned rooms that were illuminated daily from 6 a.m. to 7 p.m. Purina laboratory chow and water were available ad libitum. Mice were killed between 9 and 10 a.m. The animals were autopsied, and the stomach contents were examined to confirm that all mice were healthy and well fed during the night before death.

Biological Systems: Origin, Histology, and Biological Properties of the Human Colon Carcinoma Xenografts (2, 3)

The DLD-2 Human Colon Carcinoma Xenograft (Slowly Growing Line). The DLD-2 line was established from a well-to moderately differentiated adenocarcinoma of the human colon resected surgically at one of the Brown University Affiliated Hospitals. The original neoplasm penetrated the muscularis propria, invaded lymphatics, and was inflamed. It grew mainly as glands and had no solid areas. The glands were medium sized, well defined, and arranged in a back to back pattern; some were cystically dilated and filled with mucus. The cancer cells were large and cylindrical-cuboidal, with brush borders and mucin vacuoles. The nuclei were large and oval or round, with clefts and folds. Most nuclei contained one or more large eosinophilic nucleoli. Mucicarmine staining showed the cells to be 3 to 4+ in intracellular and intraglandular mucin, mainly supranuclear, and also along brush borders; 90% of the tumor stained positively.

An inoculum of 1 to 2 × 10⁶ cells produced palpable tumors in nude mice in about 4 to 5 weeks. The cell line in culture had a doubling time of about 48 hr and did not produce colonies in soft agar.

The Clone A Human Colon Carcinoma Xenograft (Rapidly Growing Line). The clone A cell line was derived from another surgical specimen, the DLD-1 tumor. This neoplasm was also a well-to moderately differentiated colonic adenocarcinoma with (a) small closely packed glands lining a large space, cystically dilated, filled with mucus or (b) numerous medium and small glands, back to back with prominent mucus production. The cells were of moderate to large size and of variable contour. The nuclei were medium to large and oval, with some indentation and folding. The cytoplasm was eosinophilic and frequently contained mucous vacuoles. One or more nucleoli were present in each nucleus. Mucicarmineophilic intracytoplasmic vacuoles and luminal secretions were present throughout the neoplasm.

The clone A cell line was obtained by selecting colonies growing in soft agar. The tumor produced by injection of clone A cells into athymic mice consisted of many solid nests of tumor cells. The clone A carcinoma was more pleomorphic, had an increased nuclear:cytoplasmic ratio, had an increased mitotic rate, showed decreased mucus production, and had fewer glandular structures than the DLD-1 nude mouse tumor. The clone A tumor was considered, on this basis, a poorly differentiated adenocarcinoma. An inoculum of 1 × 10⁶ cells produced tumors in nude mice in approximately 2 weeks. The cell line in culture had a doubling time of approximately 20 hr.

Development of the tissue culture cell lines, the conditions for in vitro culture, passage of cells into athymic mice, and in vitro characteristics of the cell lines were described elsewhere by Dexter et al. (2) and Hager et al. (3).

Preparation of Tumor Extracts. Clone A cells were injected 3 weeks prior to testing, and DLD-2 tumor cells were inoculated 7 weeks prior to the experiment. The tumor-bearing mice were shipped from Brown University, Providence, R. I., to the Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, Ind., and experiments were carried out 2 to 7 days later, when tumors were 1.0 cm in diameter.

The mice were stunned, decapitated, and exsanguinated. The xenograft tumors were quickly excised and placed in beakers embedded in crushed ice. The neoplasms were dissected free of macroscopically visible necrotic, hemorrhagic, and normal tissues. The careful selection and utilization of only the viable carcinomatous tissue was important in ensuring the comparability of neoplasms in the different shipments.

Biochemical Procedures

Enzyme Assays. From the tissues, 20% homogenates were prepared, and the 100,000 × g supernatant was obtained as described elsewhere (18). The resulting clear supernatant fluid was used for the enzyme assays.

The methods for determination of the enzymes of pyrimidine, purine, carbohydrate, and pentose phosphate metabolism were as cited elsewhere (9, 13–16, 18). The enzyme assays were adapted to the conditions of the colon tumors by working out optimum substrate and activator concentrations and optimum pH. As a result, enzyme measurements were carried out under linear kinetic conditions where the enzyme activity was proportionate to the amount of enzyme added and with time elapsed.

Metabolic Assays. The concentrations of ribonucleotides were measured as reported earlier (4, 5) in samples obtained by the freeze-clamp method adapted to the conditions of the tumor-bearing animals (19).

Protein content was determined by the routine method (10).

Expression and Evaluation of Results

Enzymatic activities were calculated in nmol substrate metabolized per hr per mg of protein (specific activity). Nucleotide concentrations were given in nmol/g (wet weight) of tissue. The results were subjected to statistical evaluation by means of the t test for small samples. Differences between means giving a probability of less than 5% were considered as statistically significant.

RESULTS AND DISCUSSION

Histological examination of both the slowly (DLD-2) and the rapidly (clone A) growing transplanted colon carcinoma lines...
as xenografts was done to provide a morphological characterization of the tumors on which the biochemical studies were carried out.

**Histology of the Slowly Growing Carcinoma (DLD-2) as Xenograft**

Microscopically, the tumors grew as circumscribed nodules composed of neoplastic glands producing abundant mucus and arranged on delicate fibrovascular septa. Individual cells had increased ratios of nucleus to cytoplasm, increased density of nuclear chromatin, and abnormal variability of nuclear size and shape. The cytoplasmic contours were often columnar but also were variably polygonal and ill defined. Mucus goblet cells were present; mitotic figures were seen frequently. The histopathological diagnosis was moderately differentiated adenocarcinoma with prominent mucus secretion (Figs. 1 and 2).

**Histology of the Rapidly Growing Carcinoma (Clone A) as Xenograft**

Microscopically, the neoplasm grew as circumscribed nodules consisting of sheets of malignant cells. These cells had large vesicular nuclei, often with prominent nucleoli. The cytoplasm was sparse and its borders were ill defined. The nuclei were quite crowded together and many mitotic figures were observed. A few poorly formed glands were present, identified by the peripheral location of the nuclei and central disposition of the cytoplasm of their cells. Mucicarmine-stained sections showed mucin in the lumens of some of these glands and mucin-containing vacuoles in some of the cells. The histopathological diagnosis was poorly differentiated adenocarcinoma (Figs. 3 and 4).

**Enzymology of Slowly and Rapidly Growing Human Colon Carcinoma Xenografts**

The specific activities of key enzymes of pyrimidine, carbohydrate, and purine metabolism in the slowly and more rapidly growing colon carcinosmas are compared in Table 1.

**Pyrimidine Metabolic Enzymes.** In the rapidly growing carcinoma line, the activities of the enzymes of de novo biosynthesis (CTP synthetase, orotidine 5'-monophosphate decarboxylase, orotate phosphoribosyltransferase) were markedly increased above those of the slowly growing neoplasms. The activities of the salvage enzymes (thymidine kinase, uracil phosphoribosyltransferase, uridine-cytidine kinase) were also elevated.

**Carbohydrate Metabolic Enzymes.** In the rapidly growing carcinoma, the activities of the key glycolytic enzymes (hexokinase, phosphofructokinase, pyruvate kinase) were increased above those of the slowly growing neoplasms. The activities of the salvage enzymes (thymidine kinase, uracil phosphoribosyltransferase, uridine-cytidine kinase) were also elevated.

**Purine Metabolic Enzymes.** In the rapidly growing human colon carcinoma xenografts, the activity of the first committed enzyme of de novo purine biosynthesis (glutamine PRPP amidotransferase) was increased, and concurrently the activity of the opposing, rate-limiting, purine catabolic enzyme (xanthine oxidase) was decreased. The activity of the purine catabolic enzyme (inosine phosphorylase) was also decreased. Consequently, the ratio of the activities of the synthetic to those of the catabolic purine enzymes was markedly elevated in the rapidly growing colon carcinoma line.

The activity of the enzyme that is rate limiting in channeling IMP to guanylate biosynthesis (IMP dehydrogenase) and the activity of the enzyme that produces IMP from AMP (AMP deaminase) were markedly elevated in the rapidly growing colon tumor line.

**Purine and Pyrimidine Ribonucleotides in Slowly and Rapidly Growing Human Colon Carcinoma Xenografts**

The concentrations of adenylates, guanylates, uridylates, and cytidylates were compared in freeze-clamped preparations obtained from the slowly and rapidly growing human colon carcinoma xenograft lines (Table 2). The concentrations of adenylates were higher than those of the other nucleotides. The highest concentrations were observed for ATP, whereas those for IMP, XMP, GMP, CDP, and CMP were the lowest among the nucleotides.

**Table 1**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>EC No.</th>
<th>Slow (nmol/hr/mg protein)</th>
<th>Rapid (% of slow)*</th>
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<tr>
<td><strong>Pyrimidine</strong></td>
<td></td>
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<td>Synthetic</td>
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<td>CTP synthetase</td>
<td>6.3.4.2</td>
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<td>Orotidine 5'-monophosphate</td>
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<td>decarboxylase</td>
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<td></td>
<td></td>
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<td>Orotate phosphoribosyl transferase</td>
<td>2.4.2.10</td>
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<tr>
<td>Uracil phosphoribosyl transferase</td>
<td>2.4.2.9</td>
<td>5.9</td>
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<td>Uridine kinase</td>
<td>2.7.1.48</td>
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<td>Uridine phosphorylase</td>
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<td>235.4</td>
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<td><strong>Glycolytic</strong></td>
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<td>Hexokinase</td>
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<td>Glucose-6-phosphate dehydrogenase</td>
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<td>539</td>
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<td>6-Phosphogluconate dehydrogenase</td>
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<tr>
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<td>IMP dehydrogenase</td>
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<td>Adenosine deaminase</td>
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<td>Glutamine PRPP amidotransferase</td>
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<tr>
<td>Xanthine oxidase</td>
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<tr>
<td>Inosine phosphorlase</td>
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<td>7,700</td>
<td>29</td>
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<tr>
<td>Amidotransferase:xanthine</td>
<td>0.07</td>
<td>2,592</td>
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</table>

* All values are significantly different from those of the slowly growing tumor (p < 0.05).

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7 The abbreviations used are: PRPP, phosphoribosylpyrophosphate; XMP, xanthosine monophosphate.
ties of the de novo and salvage enzymes in these metabolic pathways, respectively. The enlargement in the pools of the ribonucleotides corresponds with the elevated capacity of the purine nucleoside phosphorylase and xanthine oxidase, as demonstrated in hepatomas (5). It is striking that the expansion of the pools in rapidly growing human colon carcinoma xenografts uniformly occurred in all purine and pyrimidine ribonucleotides. This is in contrast to the elevations observed in hepatomas which occurred selectively for GMP, CTP, CMP, and the total amount of cytidylates.

Markers of Malignancy in Colon Tumor Xenografts. The progression-linked enzymic markers that might be the best indicators of the growth rate and degree of differentiation in these tumors are the activities of CTP synthetase, thymidine kinase, hexokinase, pyruvate kinase, IMP dehydrogenase, and AMP deaminase. The profound elevations observed in concentrations of XMP, UDP, CMP, and CDp should also be helpful markers in malignancy in the human colon tumors.

Biochemical Imbalance in Human Colon Tumor Xenografts: Selective Advantages Conferred and Reasons for Difficulties in Chemotherapy of These Neoplasms

The presence of a large noncycling fraction in primary human colon carcinomas contributes to the low sensitivity of these tumors to drug action. The profound biochemical imbalance observed in colon tumors throws light on some of the biochemical reasons for difficulties encountered in any single drug treatment protocol for these neoplasms. The heightened activities of both de novo and salvage pathway enzymes and the remarkably enlarged pools of purine and pyrimidine ribonucleotides should confer selective advantages for replication on the human colon carcinoma cell. Single agents that might inhibit the activity of the de novo pathway [pyrazofurin, N-(phosphonacetyl)-L-aspartic acid] could fail to provide therapeutically significant inhibition of growth (1, 12) because of the operation of the high activities of the salvage enzymes, particularly that of uridine kinase. Inhibition of CTP synthetase (by glutamine antagonists, e.g., azaserine, etc.) might be only partially effective because the marked activity of uridine-cytidine kinase should provide salvage of cytidine which is beyond the block for CTP synthetase activity. In the design of chemotherapy, an enzyme pattern-targeted approach (7, 8, 11) will have to be designed that takes cognizance of the enzymic and nucleotide pattern now revealed in these human colon tumor xenografts. For this purpose, enzymic activities and metabolic functions restricted to or characteristic of human colon mucosa cells should be identified and their behavior in neoplasia should be elucidated. Through such an approach, a selective effect of cancer drugs may be achieved that might be specific to human colon neoplasia.

The importance of the observations outlined in this investigation is emphasized by our current studies in 9 patients (17) that have confirmed, by enzymic studies of human colon mu-
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cosa and primary colon adenocarcinomas, the relevance to human primary colon tumors of the enzymic alterations reported in the present work.

ACKNOWLEDGMENT

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REFERENCES

Biochemical Programs of Human Cancer Xenografts

Fig. 1. Photomicrograph of the DLD-2 human colon carcinoma as xenograft. H & E, x 40.
Fig. 2. Photomicrograph of the DLD-2 human colon carcinoma as xenograft. H & E, x 63.
Fig. 3. Photomicrograph of the clone A human colon carcinoma as xenograft. H & E, x 40.
Fig. 4. Photomicrograph of the clone A human colon carcinoma as xenograft. H & E, x 63.
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