Ornithine Decarboxylase and Polyamines in Colorectal Neoplasia and Mucosa

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Abstract
Ornithine decarboxylase (ODC) and polyamines are intimately involved in normal cellular proliferation and are likely to play a role in carcinogenesis. ODC activity and polyamine content were measured in tissue samples obtained during colonoscopy from 48 benign neoplastic polyps (20 tubular adenomas; 28 villous adenomas), 18 cancers (including 5 malignant polyps), and adjacent mucosa. ODC activity in polyp and cancer tissue specimens was higher than in adjacent mucosa in 75 and 83% of pairs, respectively. Similarly, putrescine, spermidine, and spermine contents were higher in the majority of polyps and cancers compared to adjacent mucosa. ODC activity and polyamine content in colonic mucosa from 10 patients without a history of colorectal neoplasia that were not different from adjacent mucosal values in the patients with neoplasia. In conclusion, ODC and polyamines are elevated in the majority of colorectal neoplasms, but amounts in normal mucosa do not differentiate between patients with cancer, benign neoplastic polyps, and normal subjects.

Introduction
A variety of intracellular processes requisite for cellular proliferation and differentiation including transcription and translation regulation require polyamines (1). Intracellular concentrations of spermine, spermidine, and their precursor putrescine have in turn been shown to be highly regulated (1–5). In mammalian cells, putrescine is synthesized solely from the amino acid ornithine, although it can also be formed catabolically from spermidine in some circumstances (2). The first and generally rate limiting step in polyamine biosynthesis is catalyzed by ODC (6–8). ODC activity is low in quiescent cells, but can be decreased up to 30-fold within 4 hours by a variety of stimuli, such as feeding, after exposure to various growth factors, hormones, and drugs and after tissue injury during regenerative growth (9–11).

Several lines of evidence suggest that ODC plays an important role in carcinogenesis. Studies in tumor promotion models have shown a strong relationship between the tumor-promoting ability of carcinogens and induction of ODC activity (12–15), and inhibition of ODC activity can inhibit carcinogenesis (16, 17). A variety of tumors demonstrate increased ODC activity and polyamine content relative to normal tissue of the same organ system, including colorectal cancer compared to uninvolved normal appearing mucosa (18–31). Multiple investigators have also assessed ODC activity in benign colorectal neoplastic polyps, the putative transition lesion between normal mucosa and cancer, and have reported results varying from higher, to similar, to lower relative activity in polyp tissue versus adjacent mucosa (20–22, 27, 31–35). A variety of methodological factors may have contributed to these discrepant results, since ODC is known to be a labile enzyme and the ODC assay is very sensitive to seemingly minor technical factors (36). We report our experience using a standardized tissue procurement method (biopsy at the time of endoscopy) to assess both ODC activity and polyamine contents in colorectal neoplasia and flat mucosa.

Materials and Methods
Patients
Patients scheduled to undergo colonoscopy at the Tucson Veterans Affairs Medical Center and University of Arizona Medical Center for clinical indications gave consent for biopsy of neoplastic appearing lesions and flat mucosa from the opposite wall at the level of the lesion or from normal mucosa at the rectosigmoid junction if the patient had no history or current evidence of colorectal neoplasia. Patients with inflammatory bowel disease or a familial polyposis syndrome were excluded. The protocol was approved by the University of Arizona Institutional Review Board.

Colonoscopy was performed between 8 and 12 am and patients were fasting. Bowel preparation was performed with polyethylene glycol lavage. Colonoscopy was performed with an Olympus CF-1T10L colonoscope (Olympus Corp., Lake Success, NY) and biopsies were obtained with an Olympus FB-13U “jumbo” forceps. Biopsies from neoplastic lesions ≥1 cm in size were obtained either during colonoscopy or after a polyp had been excised with electrocautery and retrieved from the patient. Biopsies were taken from the surface of the polyp away from any apparent cautery injury. Tissue was promptly snap-frozen at the bedside and stored in liquid nitrogen until assayed within the following 4 months. All

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1 To whom requests for reprints should be addressed, at University of Arizona Health Sciences Center, Gastroenterology Section, 1501 N. Campbell Avenue, Tucson, AZ 85724.
2 The abbreviations used are: ODC, ornithine decarboxylase; DFMO, difluoromethyl ornithine.

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biopsies from an individual case were stored for identical time periods. Histological interpretation was performed by staff pathologists.

Methods

**ODC Assay.** Biopsy specimens were each homogenized in 600 μl ice-cold buffer (25 mM Tris-HCl, pH 7.5-0.1 mM EDTA-2.25 mM dithiothreitol) and centrifuged for 5 min at 10,000 g using a chilled Beckman microfuge. The supernatant was assayed within 5 min by addition of 100 μl supernatant to 150 μl buffer so that the final concentration of the reaction mixture was 15 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2.25 mM dithiothreitol, and 500 μM L-ornithine (specific activity, 1.2 mCi/mmol). After a 30-min incubation at 37°C, the reaction was stopped by addition of 0.5 ml 5 N HCl. The 14CO2 released was trapped on a filter paper containing NCS tissue solubilizer and counted after a 1-h incubation at room temperature. A DFMO-corrected blank was used for all calculations and was produced by incubating tissue supernatant with 10 mM DFMO for 10 min before assaying as above.

**Protein Determination.** Protein content was measured with bovine serum albumin as the standard and using the Read and Northcote modification of the Bradford protein assay (37). The sample homogenates were stored refrigerated until the protein assay could be performed, which was within 24 h.

**Polyamine Assay.** Polyamines were analyzed by reverse-phase, ion-paired high-performance liquid chromatography, using the method of Seiler and Knoeden (38) as applied to colorectal tissue by us (39). Briefly, tissue samples were dissected and disrupted in ODC assay buffer (see previous section on ODC analysis) and clarified at 2000 × g. The soluble fraction was made 0.2 N HClO4, and acid-soluble and -insoluble fractions were obtained by centrifugation at 12,000 × g. After addition of diaminoheptane as an internal standard, up to 200 μl of the acid soluble fraction was analyzed by high-performance liquid chromatography for polyamine contents. Using this method, the limit of detection is ~0.01 nmol for the amines putrescine, spermidine, spermine, and their acetyl derivatives. For the studies conducted here, in which tissue samples generally were prepared at a concentration which corresponded to ~1 mg/200 μl, the limit of polyamine detection was ~0.01 nmol polyamine/mg soluble protein. Protein contents were determined after solubilization in 0.5 NaOH, using the Bradford method (37).

**Histology.** Benign polyps with any component of villous architecture were categorized as villous adenomas secondary to the possibility that random sampling with endoscopic biopsies obtained a predominance of villous tissue. All other benign neoplastic polyps were designated as tubular adenomas.

Results

Sixty-six paired biopsies were obtained from a neoplastic lesion and adjacent mucosa in 55 patients (46 males; 9 females) ranging in age from 54 to 94 years (mean age, 69 years). Eighteen patients had either a cancer (n = 13) or cancerous polyp (n = 5). Of 48 benign neoplastic polyps, 20 were tubular adenomas and 28 villous adenomas. The distribution of lesions is given in Table 1. Eighty-seven % of neoplasms were located distal to the splenic flexure. Polyp sizes ranged from 1 to 5 cm.

Putrescine, spermidine, and spermine were the only amines detectable in neoplastic normal mucosa. Although spermidine and spermine were measurable in all samples, putrescine was undetectable in 30% of polyps and 15% of normal mucosa specimens.

Values of ODC activity and polyamine content in neoplastic tissue relative to adjacent flat mucosa within individuals are illustrated in Fig. 1. Tubular and villous adenomas were analyzed separately to determine if ODC or polyamines could differentiate tissues of different malignant potential. ODC and polyamine determinations in neoplasia minus normal mucosa. A positive number indicates that activity or content was higher in the neoplasm than in the normal mucosa. ODC and polyamine levels in neoplastic tissues were greater than from the mucosa in the majority of polyp-mucosa (both tubular and villous adenomas) and cancer-mucosa pairs, except for a reversed trend with putrescine content in villous adenomas relative to mucosa.

Despite a reversal in some lesion-mucosa pairs, cancer and polyp tissue overall demonstrate significantly higher ODC activity and spermidine content than adjacent mucosa when analyzed by paired t test (Table 2). Although spermine content in benign polyps was also significantly greater than adjacent mucosa, the difference between cancer tissue and mucosa did not reach significance. Conversely, putrescine concentrations were significantly higher in cancers than mucosa, but no difference was found in polyp-mucosa pairs. When analyzed collectively, mean cancer tissue ODC activity is significantly greater than in benign polyp tissue (Table 2: P = 0.01 by t test). However, mean spermine, spermidine, and putrescine content in cancers and polyps were not different. Additionally, ODC activity and polyamine content within tubular adenomas and villous adenomas were not significantly different.

Spermine and spermidine contents were highly correlated both in neoplasia (r = 0.86) and flat mucosa (r = 0.85). Modest correlations were evident between putrescine and spermine in neoplasia (r = 0.21) and mucosa (r = 0.35) and between putrescine and spermidine in neoplasia (r = 0.35) and mucosa (r = 0.33). Conversely, ODC activity was not significantly related to polyamine amounts in either tissue type (r ranging from -0.24 to 0.10) except for putrescine content in neoplasia (r = 0.64).

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**Table 1** Distribution of colorectal neplasia in 55 patients

<table>
<thead>
<tr>
<th>Location</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Descending colon</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>18</td>
<td>58</td>
</tr>
<tr>
<td>Rectum</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>100</td>
</tr>
</tbody>
</table>

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Mucosal biopsies from the rectosigmoid junction (20 cm from the anus) were obtained in 10 patients (8 males; 2 females) ranging in age from 48 to 79 years (mean age, 67 years) without a history of colorectal neoplasia who underwent colonoscopy for various clinical indications. These examinations were normal including no evidence of mucosal inflammation. This group was well matched by age, sex, and site of mucosal biopsy (80% of mucosal biopsies taken from patients with neoplasia were from the sigmoid colon or rectum) with the subgroups harbor-

Table 2  ODC activity and polyamine content (mean ± SD) in neoplastic tissue and adjacent normal mucosa

<table>
<thead>
<tr>
<th></th>
<th>ODC units</th>
<th>P</th>
<th>Putrescine</th>
<th>P</th>
<th>Spermidine</th>
<th>P</th>
<th>Spermine</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer (n = 18)</td>
<td>1.74 ± 2.43</td>
<td>0.02</td>
<td>0.17 ± 0.17</td>
<td>0.05</td>
<td>0.52 ± 0.37</td>
<td>0.02</td>
<td>0.89 ± 0.44</td>
<td>0.10</td>
</tr>
<tr>
<td>Mucosa</td>
<td>0.36 ± 0.44</td>
<td>0.09 ± 0.10</td>
<td>0.08 ± 0.08</td>
<td>0.49</td>
<td>0.56 ± 0.38</td>
<td>&lt;0.01</td>
<td>1.28 ± 0.76</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>0.77 ± 0.78</td>
<td>0.10 ± 0.13</td>
<td>0.11 ± 0.14</td>
<td>0.60</td>
<td>0.47 ± 0.35</td>
<td>&lt;0.01</td>
<td>1.03 ± 0.50</td>
<td>0.02</td>
</tr>
<tr>
<td>Mucosa</td>
<td>0.43 ± 0.78</td>
<td>0.09 ± 0.12</td>
<td>0.09 ± 0.12</td>
<td>0.47</td>
<td>0.27 ± 0.13</td>
<td>0.82 ± 0.39</td>
<td>0.60 ± 0.44</td>
<td>0.60</td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>0.67 ± 0.82</td>
<td>0.10 ± 0.14</td>
<td>0.10 ± 0.13</td>
<td>0.38</td>
<td>0.52 ± 0.37</td>
<td>&lt;0.01</td>
<td>1.17 ± 0.67</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mucosa</td>
<td>0.32 ± 0.35</td>
<td>0.10 ± 0.10</td>
<td>0.10 ± 0.10</td>
<td>0.28</td>
<td>0.28 ± 0.18</td>
<td>0.82 ± 0.44</td>
<td>0.60 ± 0.44</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*P values refer to the difference between neoplastic tissue and normal mucosa within patients (paired t-test).

One unit = 1 nmol ¹⁴CO₂/60 min/mg protein.

Nondetectable levels of putrescine were assigned a value of 0.01 nmol (the lower limit of detection); thus the actual mean values are ≤ those shown.
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Discussion

ODC activity is highly inducible and increased activity with comparatively high levels of polyamines accompanies normal cellular proliferation and differentiation (11). Enhanced ODC activity is also evident during the initiation and promotion phases of experimental carcinogenesis (12–15). A variety of neoplasms exhibit increased ODC activity relative to normal tissue of the same organ. We evaluated this relationship in colorectal tissues and also measured polyamine content, supposing increased ODC activity relative to normal tissue of the general proliferative activity. Hietala (23) has described 2- to 4-fold differences in ODC activity between multiple samples from different areas within individual tumors. We also found differences in ODC activity within biopsy samples obtained on different days from the same tumor (data not shown). Although less likely, the concept of biological heterogeneity related to ODC activity might also apply to normal-appearing colorectal mucosa, similar to focal islands of dysplasia of varying severity in the setting of ulcerative colitis defined by flow cytometry (45). Levine (46) has also described fields of hyperproliferation defined by flow cytometry in flat mucosa adjacent to some colonic neoplasms.

Several technical factors may account for some of the divergence of findings between studies. Porter (20) reported substantially lower ODC activity in both tumor and normal mucosa specimens obtained from surgical resections compared with preoperative endoscopic biopsies of the same areas. The duration between tissue resection or biopsy and tissue freezing may have been longer with the surgical specimens, an obvious confounding issue with the extremely short half-life of ODC. We have examined the effect of different methodological factors on calculated ODC activity and found significant variation dependent upon the type of buffer, the protein content of the reaction mixture, and the nature of the blank used (36). Data has been reported that ODC activity within biopsy samples significantly diminishes with prolonged storage at −20°C (47). Collectively, the multitude of potential biological and technical sources of variation in ODC activity likely account for discrepancies reported by different investigators, including the very large incongruity between ranges of ODC activity (values ranging from a few pmol to nmol CO₂/mg protein/h). Different reports by the same investigators have described ODC activity ranges varying from 1 to 100 pmol CO₂/mg protein/h.

Tissue within a neoplasm is biologically heterogeneous, with focal areas of hyperproliferation, quiescence, and perhaps necrosis accompanying varying relative amounts of epithelial, inflammatory, and stromal components (44). Hence, assaying a small fraction of a neoplastic lesion might not be representative of the general proliferative activity. Several methods have been described to overcome the problems of technical and biological variation inherent in ODC assay. McAdams and Gunz (48) have described assays using tissue homogenates or tissue sections. Using tissue sections, the enzyme protein to be assayed is applied to tissue sections and the ODC activity is assayed as previously described (9). Alternatively, the enzyme activity can be determined by measuring the incorporation of [14C]CO₂ into CO₂ after incubation of tissue sections with [14C]CO₂ for 90 min at room temperature. This method is not suitable for measuring ODC activity in clinical specimens, as the enzyme activity can be measured only if the tissue is fresh or frozen soon after harvest.

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In addition, some investigators have used radioactive labeling to assay ODC activity (49). The enzyme activity can be measured by incubation of tissue sections with [14C]CO₂ for 90 min at room temperature. This method is not suitable for measuring ODC activity in clinical specimens, as the enzyme activity can be measured only if the tissue is fresh or frozen soon after harvest.

Collectively, the multitude of potential biological and technical sources of variation in ODC activity likely account for discrepancies reported by different investigators, including the very large incongruity between ranges of ODC activity (values ranging from a few pmol to nmol CO₂/mg protein/h). Different reports by the same investigators have described ODC activity ranges varying from 1 to 100 pmol CO₂/mg protein/h. The enzyme activity can be measured by incubation of tissue sections with [14C]CO₂ for 90 min at room temperature. This method is not suitable for measuring ODC activity in clinical specimens, as the enzyme activity can be measured only if the tissue is fresh or frozen soon after harvest.

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have extended this observation to patients with sporadic colorectal neoplasia, in that ODC activity within normal mucosa was found to be significantly higher in individuals with neoplasia compared to those without these lesions (although a large overlap in ODC activity between patient groups was noted) (19, 21, 25, 32, 48, 50, 51). Mucosal polyamine content has also been reported to be significantly increased in this context (21, 30, 51). The primary implication from these studies is that elevated ODC activity reflects a presumed generalized mucosal hyperproliferation (field defect hypothesis) and might therefore serve as a marker for the presence of neoplasia elsewhere in the colon or rectum. In this context, rectal mucosal ODC activity and/or polyamine content obtained at the time of screening sigmoidoscopy could potentially be utilized to determine which patients should undergo screening colonoscopy. Although no longitudinal data has been reported to support the notion that mucosal ODC activity predicts future risk for neoplasia development (as opposed to concurrent, synchronous lesions as referred to above), a marker with this ability could be utilized to tailor future surveillance strategy for individuals (i.e., how frequently to examine the colon) and to identify appropriate candidates for intervention therapy with dietary alterations or pharmacological agents. We, however, did not find a significant difference in ODC activity or polyamine content in the flat mucosa from subjects without neoplasia compared to those harboring neoplastic polyps or cancer. Our small sample size raises the issue of a type II error; however, other investigators have also found ODC not to be discriminatory for neoplasia risk (31–34, 49, 52, 53). These divergent reports again emphasize that although most studies find ODC and polyamine content activity to be generally elevated in colorectal neoplastic tissue, substantial variability and overlap between tissues and patients exist.

Thus far colon neoplasia risk stratification utilizing tissue bioanalysis has generally concentrated on a single marker, such as mucosal crypt labeling index or ODC. As discussed above for ODC, these assays when used alone do not appear to be highly discriminatory for neoplasia risk assessment. Consequently, future efforts should attempt to use a combination of markers, perhaps together with other parameters such as diet or family history, to determine a risk profile capable of predicting the presence of neoplasia in an individual subject.

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References

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Cancer Epidemiology, Biomarkers & Prevention

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