Enhanced growth of colorectal aberrant crypt foci in fasted/refed rats involves changes in TGFβ₁ and p21CIP expressions

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We previously demonstrated that fasting/refeeding enhances the initiation phase of liver and colorectal carcinogenesis in rats. The present study was undertaken to establish whether cycles of fasting/refeeding carried out during the promotion phase of carcinogenesis may also affect the formation of aberrant crypt foci (ACF), preneoplastic lesions induced in the colon by azoxymethane (AOM). We were also interested in studying whether this effect might be mediated by changes in the proliferation, apoptosis or expression of TGFβ₁ and p21CIP genes in the colon. 44 male Fisher 344 rats were given a single dose of AOM (20 mg/kg s.c.) and one week later, they were exposed to 5 cycles of 4 days fasting followed by 7–10 days of refeeding (refed rats); controls were regularly fed; the rats were killed 2, 8 or 30 days after the last cycle of fasting. Fasting/refeeding caused a dramatic increase in crypt multiplicity when compared with regularly fed rats (ACF/ACF was 4.30 ± 1.3 in refed and 2.38 ± 0.4 in regularly fed rats, P < 0.005 means ± SD), while no significant changes were observed in the number of ACF/colon. In the two experimental groups, cell proliferation was higher in ACF than in the surrounding mucosa, but proliferative indexes were higher and the apoptotic index lower in ACF of refed rats compared with regularly fed rats. TGFβ₁ expression was higher in the ACF of refed rats than in those of fully fed controls while p21CIP was less expressed in refed rats than in controls. These results suggest that fasting/refeeding is a risk factor for colon cancer and must be taken into account for cancer prevention in humans.

Introduction

The influence of dietary habits on colorectal cancer is supported by a variety of epidemiological and experimental studies (1,2). In particular, a consistent association has been found between cancer and caloric intake: overfeeding in animals and overeating in humans being associated with a high incidence, while chronic caloric restriction is associated with a lower risk of cancer in many organs including the colon and rectum (2,3).

However, experimental studies have also demonstrated that short periods of hypocaloric diets or complete fasting followed by ad libitum refeeding enhance liver and mammary carcinogenesis (4–7). In agreement with these findings, we recently demonstrated that fasting/refeeding during the initiation phase with the carcinogen AOM increases colorectal carcinogenesis in rats (8,9). Fasting/refeeding has also been shown to increase cell proliferation in the colorectal epithelium (8,10), and high cell proliferation may increase carcinogenesis by expanding existing preneoplastic lesions.

Given these considerations and the fact that many individuals in Western countries where the incidence of colorectal cancer is high, engage unbalanced dietary habits with periods of dieting followed by binging or overeating, we wanted to study whether cycles of fasting/refeeding during the promotion phase of colorectal carcinogenesis would increase the number or growth of aberrant crypt foci (ACF) induced in the colon by azoxymethane (AOM). ACF are considered to be preneoplastic lesions in the development of colon cancer (11–13), and although it has been demonstrated that not all ACF progress to cancer (14,15), they are widely used as endpoint in short-term carcinogenesis studies to determine the chemopreventive or promoting effects of drugs or dietary factors (16,17).

We were also interested in studying whether cycles of fasting and refeeding might induce changes in cell proliferation, apoptosis and the expression of TGFβ₁ and p21CIP (genes involved in cell cycle regulation) in ACF and in the normal surrounding mucosa.

Materials and methods

Animals and treatments

Forty-four 2-month-old male F344 rats, weighing about 150 g, were used (Charles River, Como, Italy). All animals were housed in plastic cages (3–4 in each cage) in a holding room and acclimatised to a 12:12 h light-dark cycle, controlled temperature (21 ± 1°C) and humidity (70–80%) according to the European Union Regulations on the Care and Use of Laboratory Animals (18). After their arrival from the supplier, animals were quarantined for 1 week, during which they were fed a standard AIN-76 diet (Piccioni, Brescia, Italy). Rats were then given a single s.c. dose of 20 mg/kg of AOM (Sigma, Milan, Italy) and randomly allocated to one of the two experimental groups: one group was fed AIN-76 ad libitum; the other was fed the same diet but with five cycles of fasting for 4 days, followed by 7–10 days of refeeding (Figure 1). Both groups had free access to water for all the duration of the experiment. To study the proliferative activity in the colorectal mucosa, rats were killed by carbon-dioxide asphyxia at various intervals after treatment with AOM (64 days (10 rats) and 70 and 92 days (6 rats each)). The rats were injected i.p. with a single dose of 500 mCi/kg of [³H]-thymidine (NEN, Boston, MA) 1 h before they were killed.

Determination of number and crypt multiplicity of foci

The colon and rectum were removed immediately after death, flushed with 0.9% cold NaCl solution and weighed. They were slit open longitudinally and divided into three segments: proximal, median and distal. Each segment was fixed in 10% formaldehyde solution between two filter papers in a Petri dish and stained with methylene blue as described by Bird (19), to evaluate the number of ACF and crypt multiplicity. ACF were classified as small (1-2-3-4 crypts/ACF), medium (5-6-7 crypts/ACF) or large (8 or more crypts/ACF). Growth parameters and gene expression were evaluated in histological sections of ACF and the surrounding mucosa. ACF were identified under the microscope.
in the colon stained with methylene blue and marked with permanent ink (Rotring, Germany). A small colon specimen (~2 mm²) including the marked ACF was then cut with a lancet, embedded in paraffin and cut into sections (4 µm) to be mounted on slides. The samples were stained with H&E for the determination of the mitotic index, or processed for autoradiography for analysis of the labelling index. Each section was then observed under the microscope to identify the ACF, which was recognized as having a large crypt diameter often irregularly shaped. For each rat we scored at least 5 ACF which were chosen to have full longitudinal crypt sections (i.e. from the base to the bottom of the crypt) to allow the determination of the proliferation indexes and apoptosis in at least 10 aberrant crypts. In the normal mucosa, at least 10 crypts/segment of the colorectum were used to determine each mitotic and labelling indexes while apoptotic index was determined in 20 crypts/rat.

Apoptosis was detected by the TUNEL method using the ‘in situ cell death detection fluorescente’ kit (Boehringer-Mannheim, Germany) which is an enzymatic system to label in situ apoptosis-induced DNA strand breaks. In this system, the terminal deoxynucleotidyl transferase (TdT) catalyzes the polymerization of fluorescein-dUTP nucleotides to free 3'-OH DNA ends in situ. Fluorescein labels incorporated in nucleotide polymers are detected and quantified by fluorescence microscopy (Leitz, Germany). This tailing reaction using TdT is described as the TUNEL (TdT-mediated dUTP nick end labelling) technique. The number of fluorescent cells, that is the number of cells with fragmented DNA, was determined for each crypt.

For autoradiography, sections of colorectal tissue were coated with NTB-2 Kodak emulsion, dried, immersed in liquid-scintillation fluid and sealed in a dark box at ~80°C for 2 to 3 weeks. Slides were then developed and counterstained with H&E.

For each rat, we counted the number of cells on both sides of the well oriented crypts. A crypt is ‘well oriented’ when the nuclei are visible from the base of the crypt to the luminal surface. We recorded the number and the position (from the bottom to the top of the crypt) of mitotic, apoptotic and labelled cells along the crypt column. For each rat, labelling, mitotic and apoptotic indexes (%) were calculated as the number of labelled, mitotic and apoptotic cells, respectively, counted in all the crypt sections of the same rat/number of cells in all the crypt sections of the same rat × 100. The crypt column was divided into three equal parts (upper, medial and lower) to calculate the distribution of labelled, mitotic and apoptotic indexes along the crypt.

**Immunohistochemistry**

To measure the expression of TGFβ1 and p21CIP proteins we cut tissue sections 3µm thick. The sections were immersed in 0.3% H2O2 for 10 min to block endogenous peroxidase activity, incubated with sodium citrate buffer (Menarini, Florence, Italy) for 10 min in a microwave oven and incubated with goat serum (Dako, Milan, Italy) for 20 min. They were then reacted with primary mouse monoclonal antibody (Santa Cruz, CA, USA) diluted 1:50 for TGFβ1 and 1:100 for p21CIP in TBS (Tris buffer saline) for 2 h. The sections were then incubated with secondary antibody conjugated to the avidine-biotin-peroxidase complex (Dako, Milan, Italy) for 1 h. Sections were stained with 3,3′-diaminobenzidine (DAB) (Boehringer-Mannheim, Milan, Italy) and counterstained with 1% hematoxylin to score ‘positive’ cells which were identified as those with a dark brown colour.

For each rat TGFβ1 and p21CIP expressions were calculated as the number of all the ‘positive cells’ (those expressing the protein) counted in the crypts of the same rat/number of cells in all the crypt sections of the same rat × 100. As described for the proliferative and apoptotic indexes, for each rat we scored at least 5 ACF which were chosen to have full longitudinal crypt sections to allow the determination of TGFβ1 and p21CIP expressions in at least 10 aberrant crypts. In the normal mucosa, at least 10 normal crypts/rat were used to determine each parameter.

**Statistical analysis**

Data are expressed as mean ± SD; differences between groups were analyzed using Student’s t-test (n = number of rats/group). A difference was considered statistically significant when the P-value was 0.05 or below.

**Results**

**Fasting/refeeding enhances the growth of ACF**

Body weights and food intake during the experiment are presented in Figure 2 and Figure 3, respectively. Starting after the first cycle of fasting/refeeding (10 days after AOM) and throughout the course of all the experiment, body weight was lower (P ≤ 0.0001) in refed rats than in fully fed controls (Figure 2). As expected, mean food intake in refed rats was higher (P < 0.001) after each cycle of fasting when compared with regularly fed rats (Figure 3), but the total food intake of both groups was the same.

The results relative to ACF (Table I) show that the number of ACF/colon at various times after AOM administration was similar in the two experimental groups. On the contrary, when comparing the two groups 70 and 92 days after AOM (8 and
30 days after the last cycle of fast, respectively) we found that fasting/refeeding caused a remarkable increase ($P < 0.005$) in crypt multiplicity when compared with regularly fed rats.

**Fasting/refeeding increases mitotic and labelling indices and reduces the apoptotic index in the ACF of refed rats**

Since loss of the concerted regulation of cell replication and active cell death is essential for non-cancerous tissue, we measured kinetic growth parameters and apoptosis in the ACF and in the surrounding mucosa of both refed and control rats (Table I). As expected, in both groups the mitotic index was higher in ACF compared with the surrounding normal mucosa. Moreover, we found that both mitotic and labelling indexes were higher in the ACF of refed rats than in fully fed controls. The results also showed that the apoptotic index was significantly lower in the ACF of refed rats than in those of controls (Table II). The determination of the distribution of the proliferative activity and apoptosis along the crypts did not show any significant difference between groups (data not shown).

$p21^{CIP}$ expression was lower while $TGF\beta_1$ expression was higher in ACF of refed rats

Given the variations in the proliferative and cell death kinetic parameters, we also investigated whether two of the main genes involved in the regulation of cell cycle of the colorectal epithelium, $TGF\beta_1$ and $p21^{CIP}$, could mediate the influence of fasting/refeeding on ACF growth. Levels of $p21^{CIP}$ and $TGF\beta_1$ proteins were examined by immunohistochemistry in the putative preneoplastic lesions, ACF, and in the surrounding normal epithelium (Table III). $TGF\beta_1$ expression was found to be lower in ACF in comparison with the surrounding mucosa in both experimental groups, while $TGF\beta_1$ expression was higher in the ACF of refed rats than in those of fully fed controls. We also found that $p21^{CIP}$ expression was higher in the ACF than in the normal mucosa and that in both ACF and normal mucosa, $p21^{CIP}$ was less expressed in refed rats than in controls; in particular, 64 days after AOM, none of the rats in the refed group expressed $p21^{CIP}$ protein in the normal mucosa. The multiplicity of the ACF examined for protein expression ranged from a minimum of 3 AC to a maximum of 6 AC/focus. No apparent differences in protein expression were found among ACF of different multiplicity (data not shown).

**Discussion**

The results of this study indicate that cycles of fasting/refeeding during the promotion phase of colorectal carcinogenesis enhance the growth of AOM-induced ACF, increase the proliferative activity and reduce cell death by apoptosis in ACF and in normal mucosa. The results also indicate that cycles of fasting/refeeding affect the expression of $TGF\beta_1$ and $p21^{CIP}$ genes in both ACF and surrounding mucosa and that the expression of these two genes is altered in ACF as compared with normal mucosa.

Cyclic food restriction has been reported to abolish protection against mammary (5) and liver (4) carcinogenesis and more recently we showed that fasting/refeeding stimulated carcinogenesis in rat mammary glands (20) and liver (7,21,22). Contrary to these reports, other authors have shown that food restriction protect from carcinogenesis by enhancing apoptosis in preneoplastic cells (23), a finding in apparent discrepancy with the present data in which the refeeding period, after prolonged fasting, stimulates the ACF growth rate. It is possible that this stimulation might be due, in our experimental model, to nutritional factors or to the fact that fasting may have selected more aggressive ACF. Accordingly Corpet et al. (24) reported that food restriction decreased the proliferative activity in normal crypts but not in aberrant crypts induced in rats by AOM.

We also demonstrated that rats administered AOM after 4 days of fasting, developed larger ACF (8) and had a higher incidence of colorectal cancer (9). The data presented here clearly indicate that exposure of rats to various cycles of fasting/refeeding after AOM initiation, enhances crypt multiplicity of ACF without affecting the number of ACF. It is worth noting that crypt multiplicity has been proposed as the better predictor of colon cancer outcome (9,12,13). Therefore the present data demonstrate that not only the carcinogenesis initiation phase but also the promotion phase is affected by fasting/refeeding cycles. This effect was particularly striking at 92 days after AOM, when ACF multiplicity in the fasting/refeeding group

### Table I. Effect of fasting/refeeding on the number (ACF/rat) and crypt multiplicity (AC/ACF) of ACF

<table>
<thead>
<tr>
<th>Days after AOM</th>
<th>ACF/rat</th>
<th>AC/ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Refed rats</td>
</tr>
<tr>
<td>64</td>
<td>15.1 ± 5.7</td>
<td>10.15 ± 5.7</td>
</tr>
<tr>
<td>70</td>
<td>18.5 ± 7.8</td>
<td>22.2 ± 7.8</td>
</tr>
<tr>
<td>92</td>
<td>40.1 ± 8.8</td>
<td>45.1 ± 11.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (64: n = 10; 70, 92: n = 6). *P < 0.05; **P < 0.005 vs. controls.

AC, aberrant crypts; ACF, aberrant crypt foci.

### Table II. Effect of fasting/refeeding on growth parameters

<table>
<thead>
<tr>
<th>Days after AOM</th>
<th>Mitotic index</th>
<th>Labelling index</th>
<th>Apoptotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACF</td>
<td>Normal mucosa</td>
<td>ACF</td>
</tr>
<tr>
<td>64</td>
<td>Control</td>
<td>1.0 ± 0.3††</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Refed</td>
<td>1.8 ± 0.5**†††</td>
<td>0.8 ± 0.3**</td>
</tr>
<tr>
<td>70</td>
<td>Control</td>
<td>1.3 ± 0.4†</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Refed</td>
<td>1.9 ± 0.5**†††</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SD of the mitotic, labelling and apoptotic indexes in each rat of the different groups (64: n = 10 rats; 70: n = 6 rats). For each rat, labelling, mitotic and apoptotic indexes (%) were calculated as the number of labelled, mitotic and apoptotic cells, respectively, counted in all the crypt sections of the rat/number of cells in all the crypt sections of the same rat × 100. *P < 0.05; **P < 0.025; ***P < 0.0001 vs. controls. †P < 0.05; ††P < 0.025; †††P < 0.0001 vs. normal mucosa.
was almost two times higher than in regularly fed rats. It is worth noting that such ample variation in crypt multiplicity has been reported in the literature only after treatment with potent chemopreventive agents such as polyethylene glycol which is able in rats to halve the multiplicity of AOM-induced ACF when compared with controls (16).

The mechanism underlying this effect seems to involve an increase in the proliferative activity in the mucosa together with a lower apoptotic index in the refed rats, thus favouring the growth of the ACF. The enhanced growth of ACF in refed rats may be explained, at least in part, by alteration in colon cell turnover observed during the refeeding which causes a wave of proliferation associated with a slowdown of apoptosis in ACF. Refeeding after fasting has been demonstrated to increase cell proliferation in colorectal mucosa in rodents (8,10).

In our study the variations in kinetic indices and apoptosis are accompanied by changes in the expression of cell cycle related genes, such as TGFβ1 and p21CIP. TGFβ1 is the main growth inhibitory factor in normal epithelium and p21CIP is the prototype of the CIP/KIP family of cyclin-dependent kinase inhibitor (CKI) well related to TGFβ1. Indeed, the p21CIP gene has been reported to be involved in the development of colorectal cancer (43). Consistent with these reports, we found that p21CIP expression was lower in the ACF and normal colorectal mucosa of refed rats when compared with that of fully fed animals. On the other hand, we also found that, irrespective of experimental group, p21CIP expression was higher in ACF than in normal mucosa, an apparently paradoxical finding similar to that reported by us in ACF and normal mucosa of resveratrol-treated and control rats (30).

In conclusion, in the present study we demonstrate that cycles of fasting/refeeding during the promotion phase of AOM-induced carcinogenesis enhance the growth of ACF, increase the proliferation activity and decrease apoptosis in preneoplastic lesions. Since these conditions have been associated with increased carcinogenesis (44,45), these results suggest that unbalanced dietary habits characterized by periods of fasting and binge eating similarly to those represented in this paper may represent a risk factor for colon cancer, even in humans. The identification of such risk factors – which can and must be avoided – may represent one of the best strategies for cancer prevention in humans.

### Acknowledgement

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### References


### Table III. Effect of fasting/refeeding on the expression of p21CIP and TGF β1 protein levels

<table>
<thead>
<tr>
<th>Group</th>
<th>TGFβ1</th>
<th>Normal mucosa</th>
<th>p21CIP</th>
<th>Normal mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACF</td>
<td></td>
<td>ACF</td>
<td></td>
</tr>
<tr>
<td>64 Controls</td>
<td>20 ± 3.5††</td>
<td>45 ± 4.1</td>
<td>2.3 ± 0.3††</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>Refed rats</td>
<td>30 ± 3.4****††</td>
<td>57 ± 4.0***</td>
<td>1.3 ± 0.2****††</td>
<td>0.0 ± 0.0***</td>
</tr>
<tr>
<td>70 Controls</td>
<td>15 ± 2.4††</td>
<td>55 ± 3.9</td>
<td>2.2 ± 0.2†</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Refed rats</td>
<td>20 ± 2.4†††</td>
<td>58 ± 2.4†††</td>
<td>1.2 ± 0.2***†</td>
<td>0.3 ± 0.5††</td>
</tr>
</tbody>
</table>

Data are means ± SD of the % of cells expressing the two proteins in each rat of the different groups (64: n = 10 rats; 70: n = 6 rats). For each rat TGFβ1 and p21CIP expressions were calculated as the number of all the cells expressing the protein in the crypts of the rat/number of the cells in all the crypt sections of the same rat ×100. For each rat at least 10 AC and 10 normal crypts were scored. *P < 0.05; **P < 0.025; ***P < 0.0001 vs. controls. **P < 0.025; ***P < 0.0001 vs. normal mucosa.


18. European Community (1986) European Community Regulations on the Care and Use of Laboratory Animals, Law 86/609/EC.


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