Type II oestrogen binding sites in human colorectal carcinoma

M Piantelli, R Ricci, L M Larocca, A Rinelli, A Capelli, S Rizzo, G Scambia, F O Ranelletti

Abstract
Seven cases of colorectal adenocarcinomas were investigated for the presence of oestrogen receptors and progesterone receptors. The tumours specifically bound oestradiol. This binding almost exclusively resulted from the presence of high numbers of type II oestrogen binding sites. Oestrogen receptors were absent or present at very low concentrations. Immunohistochemical investigation of nuclear oestrogen receptors gave negative results. This indicates that anti-oestrogen receptor antibodies recognise oestrogen receptors but not type II oestrogen binding sites. The presence of specific type II oestrogen binding sites and progesterone binding offers further evidence for a potential role for these steroids and their receptors in colorectal carcinoma.

Evidence that female sex hormones may have a role in the subsequent development of colorectal cancer has accumulated from observations of time trends in colorectal cancer rates and from epidemiological studies. It is well known that colorectal carcinoma, has been described. Breast cancer can precede, follow, or even be synchronous with intestinal carcinomas. Furthermore, common aetiological factors have been suggested for these two cancers.

The mechanism of steroid action is generally thought to be mediated by the binding of the hormone to specific proteins termed receptors. Conflicting data, however, have been reported about the presence of oestrogen receptors in intestinal tumours. Many investigators have reported the presence of oestrogen receptors in a distinct proportion of primary colon carcinomas. Other studies, on the contrary, failed to show the presence of oestrogen receptors in colorectal cancers.

It has been shown that, in addition to high affinity, low capacity oestrogen receptors, rat and human target tissues contain a second binding macromolecule termed type II oestrogen binding site. Type II oestrogen binding sites display lower affinity but higher capacity for the ligand than oestrogen receptors. Furthermore, the presence of type II oestrogen binding sites reflects the oestrogen responsive state of tissues.

As it has been reported that the presence of type II oestrogen binding sites can interfere with the accurate quantitation of oestrogen receptors in human breast cancer and myometrium, we analysed colorectal tumor samples for the presence of both oestrogen receptors and oestrogen binding sites. Because, in target tissue, oestrogen can induce synthesis of specific cytoplasmic receptors for progesterone, we also assayed these tumours for the presence of progesterone receptors.

Methods
Specimens for hormone receptor analysis were collected at the time of surgery from the tumor mass. Samples were frozen in liquid nitrogen and stored at −80°C until use. From the samples to be assayed for receptor analysis, frozen sections for histological assessment were routinely taken to ensure that there was only a minimum of connective tissue and no normal mucosa in these. Furthermore, some of these sections were used for the immunohistochemical demonstration of nuclear oestrogen receptors with the ERIKA kit (Estrogen Receptor Immunohistochemical Assay, Abbot Laboratories, North Chicago, Illinois).

Oestrogen receptors and progesterone receptors were assayed with the use of the dextran coated charcoal (DCC) method, as described previously. Briefly, aliquots of cytosol from the homogenised, ultracentrifuged specimens were incubated with increasing concentrations of [2, 4, 6, 7,3H]-oestradiol ([3H]-E2, 101 Ci/mmol) or [3H]-Organon 2058 (60 Ci/mmol) (Amersham, Little Chalfont, England) in the presence and in the absence of unlabelled diethylstilboestrol and Organon 2058, respectively. After overnight incubation at 4°C the reaction was stopped with the addition of freshly prepared 0.4% (w/v) DCC. The mixtures were further incubated for 20 minutes at 4°C, DCC was removed by centrifugation, and the supernatant was assayed for radioactivity by liquid scintillation counting. The observed number of binding sites was expressed as femtomoles of [3H]-ligand specifically bound per milligram of cytosolic protein, and the apparent dissociation constant calculated from the Scatchard plot.

Type II oestrogen binding sites were assayed according to the procedure described by Clark and colleagues, with minor modifications. Briefly, aliquots of cytosol prepared in 10 mM TRIS-HCl, 1.5 mM EDTA, pH 7.8, at 0°C
were incubated with increasing concentrations (4-60 nM) of [6, 7-[3H]-E2 (53 Ci/nmol) alone or in the presence of a 100-fold molar excess of diethylstilbestrol at 30°C for 30 minutes, and the specific binding was determined by the hydroxyapatite method, as previously reported.19 To determine the effect that sulphhydryl reduction has on type II oestrogen binding sites cytosol was preincubated with 10 mM dithiothreitol, as reported.20

### Results

Clinical data of patients with colorectal adenocarcinoma evaluated for oestrogen receptors, progesterone receptors and type II oestrogen binding sites are reported in table 1. All female patients studied were postmenopausal. A woman was regarded as postmenopausal when periods had stopped at least one year before surgery. High affinity, low capacity oestrogen receptors were assayed using [3H]-E2 concentrations ranging from 0·1 to 3 nM and were found in three out of seven tumours, while progesterone receptors were detectable in all samples. The range of receptor concentrations was 2·5 and 7·1 fmol/mg of protein for oestrogen receptors and 7·2 and 35·9 for progesterone receptors. The dissociation constant (Kd) values ranged from 0·2 to 0·4 and from 0·2 to 0·7 nM for oestrogen receptors and progesterone receptors, respectively. When the [3H]-E2 tracer concentration was in the range 4-60 nM, a second class of binding sites appeared. In fact, the curve of [3H]-E2 binding to cytosolic samples was sigmoidal, with saturation occurring at a ligand concentration of about 45 nM (fig 1A). As predicted from the biphasic nature of the saturation curve, Scatchard analysis of the binding values yielded a concave plot (fig 1B). An accurate estimate of both the Kd and the number of type II oestrogen binding sites cannot be made from a curvilinear Scatchard plot. Reasonable estimates of these parameters can, however, be obtained from the saturation curve.19 Thus for the experiment shown, at maximum binding, the amount of type II oestrogen binding site was 1·040 fmol/mg of protein. The Kd value determined from the [3H]-E2 concentration required for half saturation was 24 nM (fig 1A). Hill analysis of binding data yielded coefficients greater than 2 (fig 2). A reduction in specific binding of about 70% was observed in the presence of 10 mM dithiothreitol. As indicated in table 1, the neoplastic tissues from all patients express type II oestrogen binding sites. The amount of these sites ranged from 273 to 1040 fmol/mg of protein, with a Kd value of 20-3 (2·0) nM (mean (SD) of seven cases). Experiments for steroid specificity showed that both oestrogen receptors and type II oestrogen binding sites are oestrogen specific. Only those steroids with oestrogenic activity inhibited the binding of [3H]-E2, whereas non-oestrogenic steroid did not (table 2, and data not shown). All seven cases, when examined with the immunohistochemical technique, were oestrogen receptor negative.

### Table 1 Oestrogen and progesterone receptors and type II oestrogen binding sites in colorectal carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Site</th>
<th>Duke's staging</th>
<th>Oestrogen receptors</th>
<th>Progesterone receptors</th>
<th>Type II oestrogen binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Rectum</td>
<td>A/well</td>
<td>2·5±0·2 (0·2)</td>
<td>19·5 (0·3)</td>
<td>358 (18)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>Sigmoid</td>
<td>B/moderate</td>
<td>3·5±0·4 (0·7)</td>
<td>35·9 (0·7)</td>
<td>273 (20)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>Sigmoid</td>
<td>B/moderate</td>
<td>0</td>
<td>12·7 (0·4)</td>
<td>1040 (24)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Cecum</td>
<td>C/moderate</td>
<td>0</td>
<td>7·2 (0·4)</td>
<td>367 (19)</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>Rectum</td>
<td>B/moderate</td>
<td>0</td>
<td>13·8 (0·2)</td>
<td>479 (21)</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>Sigmoid</td>
<td>D/proo</td>
<td>0</td>
<td>8·3 (0·4)</td>
<td>712 (21)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>Ascending</td>
<td>C/moderate</td>
<td>5·3 (0·3)</td>
<td>22·1 (0·5)</td>
<td>412 (19)</td>
</tr>
</tbody>
</table>

*Results are expressed as fmol/mg of protein. The corresponding nanomolar values of the dissociation constant (Kd) are in parentheses.

### Figure 1

(A) Specific binding of [3H]-E2 as a function of tracer concentration in colorectal adenocarcinoma. (B) Scatchard analysis of data from (A).

### Table 2 Steroid specificity of type II oestrogen binding sites

<table>
<thead>
<tr>
<th>Competing steroids</th>
<th>Case 1</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Progesterone</td>
<td>89</td>
<td>84</td>
</tr>
<tr>
<td>5alpha-Dihydrotestosterone</td>
<td>98</td>
<td>103</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td>Cortisol</td>
<td>91</td>
<td>95</td>
</tr>
</tbody>
</table>

*All competing steroids were 100-fold molar excess relative to [3H]-E2 tracer concentration (40 nM). Results are expressed as percentage of binding in the absence of competitors.
Discussion

Colorectal carcinoma cytosol preparations are able to bind oestrogens specifically. This binding activity almost exclusively results from the presence of high concentrations of type II oestrogen binding sites, whereas oestrogen receptors are absent or present at very low (<10 fmol/mg of protein) concentrations. The concave Scatchard plot and the Hill coefficient of more than 2 for type II oestrogen binding sites suggest that they are multiple and that they display positive cooperation. Another distinguishing characteristic of type II binding—is its sensitivity to the presence of sulphhydryl-reducing reagents such as diisothreitol—is displayed by oestrogen binding sites in colorectal carcinoma.

The presence of high numbers of this second binding site can interfere with the accurate quantitation of oestrogen receptors. This is particularly true when an inappropriate range of tracer concentrations is used to assay oestrogen receptors—that is, at concentrations higher than 3 nM of the [3H]-E2 tracer, in addition to oestrogen receptors, also binds to type II oestrogen binding sites. Colorectal carcinomata are characterised by high numbers of type II oestrogen binding sites but are unreactive when tested with the anti-oestrogen receptor antibody. This indicates that commercially available anti-oestrogen receptor antibody recognises oestrogen receptors but not type II oestrogen binding sites.

Type II oestrogen binding sites have been found in both normal and neoplastic rat and human "classic" target tissues,11,21 human peripheral blood mononuclear cells,12 acute lymphoid and myeloid leukaemias,15 and rat15 and human pancreas,17 but their role is still unknown. In this respect it has been shown that there is a close relation between increased numbers of these sites and true uterine growth.18 Moreover, in peripheral blood mononuclear cells type II oestrogen binding sites are modulated by oestrogen.19 There is also a positive correlation between the numbers of type II oestrogen binding sites and concentrations of progesterone receptors.19 From these data it seems likely that type II oestrogen binding sites constitute an important aspect of oestrogen sensitivity. On the other hand, it may be argued that these sites in normal and neoplastic tissues have no physiological importance because of their apparent low affinity for oestrogens and the low circulating oestrogen concentrations. It is possible, however, that high concentrations of oestrogens exist within tissues or subcellular compartments. Binding sites need not be saturated to be functional: oestrogen bound to a small number of sites (spar receptors) can be effective in hormone action.

Our results indicate that in colorectal cancer the absence of oestrogen receptors does not necessarily preclude the presence of high affinity progesterone receptors. This finding agrees with the observation that oestrogen treatment is not needed to stimulate the production of progesterone receptors in chicken intestinal mesothelium and smooth muscle.24

In conclusion, the presence of type II oestrogen binding sites rather than that of oestrogen receptors could provide support for earlier suggestions25,26 that there is a female sex hormone component in the risk profile for colorectal cancer.

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