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In vitro Growth Ability and Chemosensitivity of Gastric and Colorectal Cancer Cells Assessed with the Human Tumour Clonogenic Assay and the Thymidine Incorporation Assay

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A human tumour cloning assay (HTCA) has been performed on 191 samples of gastric and 152 samples of colorectal cancers, and a thymidine incorporation assay (TIA) on 178 samples of gastric and 109 samples of colorectal cancers. The rate of evaluable assays was significantly higher in the TIA than in the HTCA ($P < 0.01$). In terms of *in vitro* growth potential in the two assays, gastric cancer cells were less active than the colorectal cancer cells ($P < 0.05$). In frequency of *in vitro* sensitivity to drugs, gastric cancer was more chemosensitive than colorectal cancer in both assays. The *in vitro/in vivo* correlations of high resistance-predictive ratios and low sensitivity-predictive ratios were similar in both assays. The results indicate that the TIA is more applicable than the HTCA to screening of active agents against fresh gastrointestinal cancers.
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CHEMOSENSITIVITY ASSAYS using tetrazolium dyes (MTT assay) have been used in the field of haematological malignancies [1–3] and a modified form of this is currently being successfully applied by the National Cancer Institute USA to the chemosensitivity testing of new drugs on cell lines [4]. However, when it is applied to solid tumours like gastrointestinal cancers, a formazan product from viable fibroblasts and other non-malignant cells cannot be excluded at the assessment [5].

The human tumour clonogenic assay (HTCA) using a soft agar culture system independently developed by Hamburger and Salmon and by Courtenay *et al.* has been shown to be suitable for culturing a variety of solid tumours [6, 7]. In this study, the HTCA was based on the method of Hamburger and Salmon. In spite of the early favourable reports of the HTCA, its practical value for predictive testing has been the focus for debate, chiefly because of the insufficient rates of evaluable assays [8–16].

To attain high evaluability rates we developed the thymidine incorporation assay (TIA) in which a two-layer soft agar culture system was used as well as in the HTCA [17–21]. In addition, a wide range of assay evaluability rates for different tumour types have been observed [10], which means that individual assay evaluability of tumour specimen should be studied on the selected tumour. In this study, therefore, the organs with tumour studied have been restricted to the stomach and the large bowel, and their assay evaluabilities and chemosensitivities were comparatively assessed with the HTCA and the TIA.

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MATERIALS AND METHODS

Tumour collection

Solid tumour specimens were obtained by surgery and immediately placed in Chee's modification of essential medium (CEM)(MA Bioproducts, Walkersville, USA) supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin (both from Gibco) and 15% heat-inactivated fetal calf serum (Flow).

Cell suspensions were prepared enzymatically [13, 17]. There were no cases where multiple samples were taken from the same tumour.

HTCA

Cells were cultured as described [13]. Colonies were defined as clusters greater than 60 μm in diameter. A maximum number of colonies per dish was reached within 3–4 weeks and counted with an automatic particle counter SP-3000 (Shiraimatsu, Osaka). 1 ml 10% formalin was poured over the top layer of one plate and the preparation was stored at 4°C. This plate was counted on the day of colony counting to obtain the background colony counts. Experiments were valid if the net number of colonies in the untreated controls was greater than 50; the background colony count was less than 25 "colonies" or clumps; and the mercuric chloride (100 μg/ml) treated dishes showed at least 70% inhibition of colony formation when compared with the controls.

TIA

Assays were plated as in the HTCA, but agarose (Seaplaque, FMC, Rockland, USA) was used instead of agar. After 72 h, 5 μCi of tritiated thymidine (specific activity, 2.0 Ci/mmol; New England Nuclear, Boston) were layered over each dish and the plates were returned to the incubator for an additional 24 h. Incorporation of thymidine by tumour cells was measured as described [17]. An assay was valid if the average count of the untreated controls was greater than 500 cpm, and the mercuric chloride control showed at least 80% inhibition compared with the untreated control.

In vitro *drug exposure*

Drug solutions were prepared from standard intravenous formulations at the concentrations (μg/ml) indicated: doxorubicin (0.4), bleomycin (2.0), 5-fluorouracil (5-FU) (10.0), mitomycin (1.0), melphalan (1.0), and cisplatin (2.0).

Clinical correlations of in vitro *results*

In vivo sensitivity was defined based on the criteria described previously [9, 17]. Patients achieving clinical responses when treated with two or more drugs that were active *in vitro* were considered to have responded only to the most active *in vitro* agent. Conversely, patients with no responses who received multiple agents that had no *in vitro* activity were considered to have true negative correlations to each agent administered.

RESULTS

Assay evaluabilities in the HTCA and TIA

Valid assays in the HTCA were achieved in 30% (58/191) of gastric cancer specimens and in 46% (70/152) of colorectal cancer specimens, whereas in the TIA 43% (77/178) of gastric cancers and 56% (61/109) of colorectal cancers. The rate of valid assays was higher in the TIA than in the HTCA ($P < 0.01$). In addition, gastric cancer cells produced significantly less clones in the HTCA than colorectal cancer cells ($P < 0.005$) and incorporated less tritiated thymidine in the TIA ($P < 0.01$).

Drug sensitivity studies

Tables 1 and 2 detail the *in vitro* sensitivity to the six anticancer drugs tested. As a definition of sensitivity to anticancer drugs ≥ 50% inhibition of colony formation in the HTCA and ≥ 80% inhibition of thymidine incorporation in the TIA were used [9, 19]. When comparing the frequencies of *in vitro* sensitivity with various drugs, gastric cancer (32.6% (95/291) in the HTCA and 26.7% (71/266) in the TIA) was more chemosensitive than colorectal cancer (28.3% (102/363) in the HTCA and 19.6% (41/209) in the TIA). When *in vitro* sensitivities to an individual drug were assessed, cisplatin was more active against gastric cancer than colorectal cancer both in the HTCA and the TIA ($P < 0.01$).

Table 1. In vitro *sensitivity to various drugs in the HTCA*

Drug	Gastric cancer	Colorectal cancer
Bleomycin	14/ 35 (40)	11/30 (37)
Cisplatin	18/ 44 (41)	19/64 (30)*
Doxorubicin	15/ 57 (26)	17/77 (22)
5-fluorouracil	24/ 63 (38)	27/80 (34)
Melphalan	3/ 25 (12)	5/28 (18)
Mitomycin	20/ 68 (39)	23/84 (27)
Totals	95/291 (32.6)	102/363 (28.3)

Sensitive/tested (%).
*$P < 0.01$, gastric versus colorectal cancer.

In vitro/in vivo *correlations*

In the HTCA, in which tumour found to be resistant *in vitro*, there were 2 clinical responses found [95% (36/38) prediction accuracy for resistance], while there were 13 clinical responses in 25 instances where the tumour was assayed as sensitive *in vitro* (52% (13/25) prediction accuracy for sensitivity). In the TIA, 40% (8/20) prediction accuracy for sensitivity and 80% (21/24) prediction accuracy for resistance were found (Tables 3 and 4).

DISCUSSION

Gastric cancer cells grew less well in both the HTCA and TIA than colorectal cancer cells. There were wide variations in *in vitro* growth ability among tumours from the different organs. Growth in ovarian cancer, melanoma and lung cancer was good, whereas growth ability for lymphoma, multiple myeloma and osteosarcoma was lower [8].

The relation of the *in vitro* growth ability to the nuclear differentiation of the tumour biopsy has been discussed for several tumours. The issue is, however, debatable. Whilst some noted that well differentiated breast cancer formed fewer

Table 2. In vitro *sensitivity to various drugs in the TIA*

Drugs	Gastric cancer	Colorectal cancer
Bleomycin	3/ 16 (19)	1/ 13 (8)
Cisplatin	15/ 40 (38)	3/ 36 (8)*
Doxorubicin	10/ 51 (20)	8/ 43 (19)
5-fluorouracil	19/ 69 (28)	15/ 54 (28)
Melphalan	1/ 10 (10)	0/ 9 (0)
Mitomycin	23/ 80 (29)	14/ 54 (26)
Totals	71/266 (26.7)	41/209 (19.6)

Sensitive/tested (%).
*$P < 0.01$, gastric versus colorectal cancer.

Table 3. In vitro–in vivo *correlations with the HTCA*

	Drug comparisons	*In vitro/in vivo* S/S	S/R	R/S	R/R	TPR (%)	TNR (%)
Gastric cancer	29	8	5	1	15	62	94
Colorectal cancer	34	5	7	1	21	42	95
Totals	63	13	12	2	36	52	95

TPR = true positive rate; prediction accuracy for sensitivity.
TNR = true negative rate; prediction accuracy for resistance.
S = sensitive; R = resistant.

Table 4. In vitro–in vivo *correlations with the TIA*

	Drug comparisons	*In vitro/in vivo* S/S	S/R	R/S	R/R	TPR (%)	TNR (%)
Gastric cancer	25	5	7	3	10	42	77
Colorectal cancer	19	3	5	0	11	38	100
Totals	44	8	12	3	21	40	88

colonies than the poorly differentiated tumours, Nomura *et al.* in a study on breast cancer and in our study on colorectal cancer found no relation of *in vitro* clonogenicity with histology [22–24]. Also, we have demonstrated that well-differentiated gastric cancer formed inversely more colonies than poorly differentiated tumours [25]. Thus, tumour histology seems not necessarily related to growth and/or chemosensitivites *in vitro*.

Gastric cancer was significantly more sensitive to cisplatin than colorectal cancer in both assays. Cisplatin has been evaluated as one of potential drugs against gastric cancer, but not against colonic cancer. Combination regimens including cisplatin, such as EAP (etoposide/doxorubicin/cisplatin) and FAP (5-FU/doxorubicin/cisplatin), have achieved better results than the regimens used more frequently like FAM (5-FU/doxorubicin/mitomycin) [26]. However, this study showed that no drugs except cisplatin had significantly different activities against the two types of tumour.

Results of clinical correlations with HTCA and TIA were analysed for each tumour type. Resistance-predictive ratios were always satisfactorily high, regardless of the type of tumour. Conversely, the prediction accuracies for sensitivity were around half in each assay. Today, gastric or colorectal cancer seems to respond partly to chemotherapy. Single agent chemotherapy induces remission in only 20% of cases or less [27]. These correlation data, therefore, can be considered to effect the clinical background that there are not enough effective agents available against gastrointestinal cancer.

Our study with fresh gastrointestinal cancer specimens demonstrated that the TIA achieved significantly higher evaluable rates than the HTCA, preserving the predictive potential of clinical chemotherapy responses. Therefore, when comparing the two assays for screening of active drugs against fresh gastrointestinal cancers, the TIA is more useful, because results are obtained more rapidly (5 days vs. 2–4 weeks), fewer tumour cells are required for testing and chemosensitivity results are evaluable in a higher number of specimens.

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Heterogeneity of Oestrogen Receptor Expression in Normal and Malignant Breast Tissue

K.J. Walker, R.A. McClelland, W. Candlish, R.W. Blamey and R.I. Nicholson

The heterogeneity of oestrogen receptor (ER) expression has been examined in both normal and malignant breast tissue using an immunohistochemical assay. In both instances the ER status and cellular ER negativity were influenced by the patients' menopausal status, with tissues removed from premenopausal women being more often ER-negative, and when ER-positive, containing a high proportion of apparently ER-negative cells. Since the breast is normally regarded as hormone sensitive and since tumour cell ER negativity is apparently under a degree of hormonal regulation, our results suggest that the proportion of breast cancer cells that are ER-negative should be viewed with a degree of caution.
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INTRODUCTION

IMMUNOCYTOCHEMICAL STUDIES on the distribution of oestrogen receptors (ER) in breast cancer specimens have revealed considerable heterogeneity of receptor expression [1, 2]. These tumours may show variation in the quantities of antibody-detected receptor per cell and in the ratio of positive to apparently negative cells. Clinically, these observations are of potential interest since whole tumour receptor negativity has previously been equated with hormone insensitivity and would imply that apparently negative cells within ER-positive breast cancers might also carry with them a poor prognosis for the patient. Indeed, preliminary data from this laboratory have tended to support this notion, with high rates of ER negativity in breast cancer cells being linked to an increased likelihood of a tumour being poorly differentiated, fast growing [1] and hormone-insensitive [3].

Since the therapeutic implications of equating cellular ER negativity in overtly ER-positive tumours with tumour cell autonomy are far reaching and might include combining endocrine and cytotoxic therapeutic regimens, it is essential to eliminate all alternative explanations before clinically acting on this new information. In this light we have undertaken a comparative investigation of the distribution of ER in normal and malignant breast tissues under the physiological influence of the menopause. Our data justify a cautious approach to the interpretation of tumour cell ER negativity and highlight the need for additional markers of hormone sensitivity and independence.

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PATIENTS AND METHODS

218 breast tissue sections from patients of known menopausal status who presented with either primary breast cancer (City Hospital, Nottingham), benign disease (Royal Infirmary, Glasgow) or for reduction mammoplasty (St Lawrence Hospital, Chepstow) were analysed for the presence of ER using the Abbott ER-ICA [1]. Tissue biopsies were trimmed of fat and connective tissue and immediately frozen in liquid nitrogen and stored at −70°C. Specimens were processed according to the Abbott ER-ICA monoclonal kit (Abbott Laboratories, North Chicago, Illinois).

Briefly, tissue sections were cut at 5 μm thickness at −20°C and mounted onto glass slides coated with tissue adhesive. Tissues were fixed by placing them in 3.7% formaldehyde in phosphate-buffered saline (PBS) (v/w) solution for 15 min. The slides were then rinsed in PBS for 5 min and stored in ER-ICA specimen storage medium at −20°C for up to 1 month before assay. At the time of assay the specimens were first washed in PBS prior to the addition of blocking agent (normal goat serum). After incubation for 15 min in a humidified chamber, excess blocking agent was removed. The primary antibody (monoclonal rat anti-human ER H222) was added dropwise to one slide of each specimen and incubated for a further 30 min. Non-specific staining was controlled using parallel sections incubated with an inappropriate normal rat immunoglobulin. Slides were washed twice in PBS for 5 min before incubation with first the bridging antibody (goat anti-rat IgG) for 30 min followed by rat peroxi-