Pergamon

European Journal of Cancer Vol. 31A, No. 1, pp. 85–91, 1995

0959-8049/95 $9.50+0.00

0959-8049(94)00377-7

Radiosensitivity of Multicellular Tumour Spheroids Obtained from Human Ovarian Cancers

G. Griffon, C. Marchal, J.-L. Merlin, S. Marchal, R.M. Parache and P. Bey

The radioresponsiveness of immunologically characterised (KL_1, antivimentin and OC125) human ovarian carcinoma cells, obtained from effusions or solid tumours, was assayed *in vitro* using the multicellular tumour spheroids (MTS) three-dimensional model. Great interspecimen variabilities were observed in MTS doubling time (1.0–8.5 days), as well as in the doses inducing a 50% decrease in the MTS individual volume (ID_{50}) (0.56–9.15 Gy), or in the overall population MTS number (SCD_{50}) (1.9–15.7 Gy) and the residual/initial MTS individual volume ratio after 2 Gy irradiation (RSV_2) (10–88%). The doubling time, DNA-ploidy and S-phase fraction did not correlate with the ID_{50}. Significant correlations were found between the new parameters defined (RSV_2 and ID_{50}) and the SCD_{50}, a well-accepted local control parameter. These parameters demonstrated their usefulness for studying the radiosensitivity of MTS prepared from human ovarian tumour biopsies.

Key words: multicellular tumour spheroids, radiosensitivity, ovarian cancers, primary cultures, OC 125 marker
Eur J Cancer, Vol. 31A, No. 1, pp. 85–91, 1995

INTRODUCTION

THE ROLE of radiation therapy in the treatment of ovarian cancer has been the subject of considerable controversy over the last decades. It has been demonstrated that pelvic irradiation is not always effective in ovarian cancer, since relapses may occur inside the whole peritoneal cavity [1]. Until the 1960s, postsurgery radiotherapy was the only treatment for ovarian cancers. However, studies have shown that it can be efficient only if the size of residual lesions after surgery is less than 2 cm. If above 2 cm, a curative radiotherapeutic treatment cannot be applied because of the occurrence of dramatic abdominal side-effects. If below 2 cm, radiation therapy can be envisaged, but abdominal side-effects must be taken into account [2]. Therefore, there is considerable interest in examining the role that tumour radiosensitivity may play in treatment response. The relation between the inherent radiosensitivity measured *in vitro* and the *in vivo* sensitivity of tumours has recently been demonstrated with animal tumour models of fibrosarcoma, melanoma, leukaemia, and mammary sarcoma [3]. These assays, which accurately measure the inherent radiosensitivity, may significantly influence the design of individualised cancer therapy. However, the clinical relevance has still to be conclusively established.

The present study aims at evaluating the radiosensitivity of ovarian microtumours using an *in vitro* test based upon the response of the three-dimensional cell system. Recently, Hoffman and colleagues [4–6] proposed three-dimensional histoculture on collagen sponge gels for predictive assays of chemotherapeutic response and evaluation of new cancer drugs. However, because this tumour model has not yet been tested for radiobiological purposes, and in order to compare our results with those already reported, we have selected the spheroid model initiated by Sutherland and associates [7].

The multicellular tumour spheroid (MTS) is a three-dimensional multicellular model that grows from established tumour cell lines or, less frequently, directly from primary tumour specimens. This tumour model is intermediate in complexity between standard two-dimensional monolayer cultures *in vitro* and tumours grown *in vivo*, and it has been reported to 'simulate micrometastasis or intervascular microregions of larger tumours' [8], with diffusion gradients for oxygen and glucose or other nutrients resulting in necrotic areas containing hypoxic cells at acid pH, which present a wide pattern of radiosensitivity [8–12]. Changes in the radiosensitivity of cells in MTS were demonstrated to be due mainly to intercellular contact, cell cycle redistribution and hypoxia [8]. Each of these variables was studied separately by comparing the radiation response of spheroids grown to different sizes and single cells isolated from MTS or grown as monolayers [3, 13–15]. Cells growing in close intercellular contact during the colony formation phase had an enhanced capacity to accumulate and to repair sublethal radiation damages [16], and exhibited an additional increase in radiation resistance [15].

To our knowledge, only a few experiments have been reported on MTS obtained from surgical specimens of human ovarian tumours. Based on our 10 year experience of primary culture from human tumours [17], producing 105 different tumour samples from 63 effusions and 42 solid tumours, we observed the highest frequency of colony formation for breast and ovarian cancers. For this reason, we studied the feasibility of radiosensitivity testing on MTS obtained from ovarian tumours.

Correspondence to G. Griffon.
G. Griffon, J.-L. Merlin and S. Marchal are at the Laboratoire de Recherche en Oncologie; C. Marchal and P. Bey are at the Service de Radiothérapie; R.M. Parache is at the Service d'Anatomie Pathologique et de Cytologie, Centre Alexis Vautrin, 54511 Vandoeuvre-les-Nancy, France.
Revised 11 Jul. 1994; accepted 7 Sep. 1994.

MATERIALS AND METHODS

Preparation of tumour specimens

8 solid tumours, 9 ascitic fluids and 1 pleural effusion were obtained from 18 patients with ovarian cancer undergoing laparotomy. 2 patients had been treated previously by chemotherapy and/or radiotherapy. Immediately after surgery (less than 30 min), the samples were transported to the laboratory in Ham's F12 culture medium (Biochrom, Berlin, Germany). The solid tumour specimens were finely chopped and incubated for 1 h at 37°C in an enzymatic solution containing Type II collagenase (Sigma, Saint-Quentin, Fallavier, France), pronase and Grade II DNase (Boehringer Mannheim, Meylan, France) at concentrations of 0.2 mg/ml, 0.5 mg/ml and 0.2 mg/ml, respectively, in Ham's F12 medium supplemented with 10% inactivated fetal calf serum (Dutscher, Brumath, France), penicillin (500 Ul/ml), gentamicin (5 μg/ml), hydrocortisone (0.5 mg/ml), transferrin (2.5 μg/ml), epidermal growth factor (10 μg/ml), insulin (10 mg/ml) and glutamine (0.3 mg/ml).

When incubation was completed, the tumour fragments and the cells were filtered through a 46 μm polyester membrane then washed in the culture medium. Ascitic fluids and pleural effusion underwent only centrifugation (300 **g**, 10 min) to collect the cells. Contaminating erythrocytes were eliminated after centrifugation of the cells on Ficoll–Paque® (Pharmacia LKB, Les Ulis, France). Viability was assessed using the trypan blue dye exclusion test.

Immediately after disaggregation of each tumour or centrifugation of each effusion, the morphological characterisation of harvested cells were performed by May–Grunwald–Giemsa [18] and Papanicolaou stainings.

Immunological analysis

Three monoclonal antibodies were used to characterise the cells harvested from ovarian tumours: anticytokeratin KL_1 (Immunotech, Marseille, France), antivimentin (Boehringer Mannheim) and OC125 (CisBio, Gif-Sur-Yvette, France). Classically, the anticytokeratin is considered to be a specific marker of epithelial cells, antivimentin a specific marker of mesothelial cells and OC125 a marker of ovarian epithelial tumours. The immunological characterisation of cells was performed using immunocytochemistry and flow cytometry (Orthocyte® flow cytometer, Ortho Diagnostic Systems, Roissy, France).

As soon as cells were disaggregated, they were smeared on slides, dried and stored at −80°C. Immunocytochemistry was then performed using the streptavidin–biotin–peroxidase complex (LSAB kit, Dako, Trappes, France) according to Wood and colleagues [19]. Briefly, the primary antibodies were applied appropriately diluted in phosphate-buffered saline. After washing, the slides were incubated with biotinylated mouse anti-rabbit immunoglobulins and rinsed. Then, the streptavidin–biotin–peroxidase complex was added and the peroxidase reaction was initiated by the addition of amino-ethylcarbazol. After rinsing, the slides were counterstained with Harris' haematoxylin and mounted under a coverslip with Immu-mount (Shandon, Paris, France). As a positive control, cells of tumour known to be strongly reactive with the corresponding monoclonal antibody were included in each staining procedure. Negative controls were performed by omitting the primary antibody.

The flow cytometry analyses were performed using the same monoclonal antibodies. The cells (10^6 cells/ml) were fixed in a mixture containing 1 ml RPMI 1640 culture medium, 1 ml fetal calf serum and 6 ml cold 70% ethanol and kept at 4°C. Then, after washing, the cell suspensions (200 μl) were incubated for 30 min at 4°C with monoclonal antibodies (20 μl). The final concentrations used were 4 μg/ml for KL_1 and 10 μg/ml for antivimentin and OC125. The primary antibodies were conjugated to fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG serum (Sigma) for 30 min at 4°C. The excitation wavelength was of 488 nm, emission was recorded at 520 nm.

Cell cycle analysis

Aliquots of the cell suspensions obtained after disaggregation of solid tumour or centrifugation of effusions were immediately fixed in cold 70% ethanol. DNA ploidy analysis of each cell suspension was performed within one week by flow cytometry after staining of nuclei with propidium iodide (Sigma) according to Vindelov and colleagues [20]. The percentage of cells in the individual cycle phases was calculated from the fluorescence data using Dean and Jett's model from Multicycle® software (Phoenix, San Diego, California, U.S.A.).

Multicellular tumour spheroids (MTS)

The cells collected from patients' specimens were plated using a modified method of Yuhas and colleagues [21]. Six-well plates (35 mm dishes) were coated with 1 ml of 0.5% agarose (Prolabo, Paris, France) in order to inhibit the growth of fibroblasts, received 2 ml of cellular suspension containing 10^4 to 10^5 cells/ml and were incubated at 37°C, in 5% CO_2. 4–5 days later, small spheroids developed that could be harvested individually using a Pasteur pipette and seeded in 24-well plates coated with 0.5% agarose and tested for radiosensitivity. The culture medium was changed twice weekly, and the growth of MTS was determined by measuring, twice weekly, the diameter using an inverted miscroscope fitted with an ocular micrometer. The diameter measured was then converted into volume, assuming spherical geometry. MTS growth curves were drawn by plotting the relative volume variations (volume (V)/initial volume ($V0$)) versus time after treatment. The size homogeneity of the MTS population analysed for radiosensitivity was ensured by only selecting MTS whose individual diameters were close to the mean diameter (±SD) of the whole MTS population formed from each individual tumour specimen.

Irradiation procedure

A Theratron 780C $^{60}C0$ unit (Theratronics) operating at 1.25 MeV was used for irradiation. Spheroids were irradiated (0–8 Gy) at a dose rate of 5 Gy min^{-1}. The suspensions were kept in 24-well plates at room temperature during exposure. Control plates were removed from the incubator for the same period of time, but were not irradiated.

Estimation of response to radiation

Seven days after irradiation, the residual/initial MTS individual volume ratio after 2 Gy irradiation (RSV_2) and the dose inhibiting the MTS growth by 50% (ID_{50}) were calculated mathematically from the fitted dose response curves constructed by plotting the normalised ratio of Virradiated/Vcontrol versus the irradiation dose.

MTS were considered 'cured' when they 'shrank' at D7 after irradiation. The 'cured' proportion of MTS was calculated as the fraction of 'shrunk' spheroids relative to the total number of spheroids originally present in the treated population. Then, spheroids control curves were plotted and the dose required to reduce the number of MTS by 50% as compared with the control

(SCD_{50}) according to Stuschke and colleagues and Schwachöfer and colleagues [3, 22] was calculated mathematically.

Statistical analysis

Statistical significance was tested using a linear regression by Fisher's test.

RESULTS

Multicellular tumour spheroids (MTS)

Eighteen samples were put into culture: eight solid tumours, corresponding to two endometrioid adenocarcinomas, two mucinous cystadenocarcinomas, one serous cystadenocarcinoma and three serous adenocarcinomas; nine ascitic fluids including one from a mucinous adenocarcinoma, six from serous adenocarcinomas, one from a cystadenocarcinoma, one from a mullerian carcino-sarcoma, and one metastatic pleural effusion from an 'ovarian carcinoma'. Eight cell suspensions formed MTS within 7 days after they were put into culture, i.e. 44% of the samples (Table 1) representing 50% of the effusions (5/10) and 38% of the solid tumours (3/8). The others formed only small cell clusters that could not be evaluated. Samples obtained from mucinous ovarian adenocarcinomas never formed MTS (0/3). Effusions were more productive (5/10) than disaggregations of solid tumours (3/8) (Table 1).

Immunological analysis

Seven of the eight samples that formed MTS were analysed using flow cytometry. The results (Table 2) showed relatively notable labelling rates with KL_1 (12 to 80%) and antivimentin antibody (20 to 64%). OC125 labelling was less (0 to 34%). Some mesothelial cells were also labelled with the OC125, although it is classically specific of epithelial ovarian tumour cells.

When plated again in liquid medium, the MTS obtained from sample 18 remained labelled as in the original cell suspension. Reading of the slides after standard staining (Papanicolaou and May–Grunwald–Giemsa) confirmed the absence of mesothelial cells.

Cell cycle analysis

DNA-ploidy was measured in five cases (Table 2) with only one case (20%) of DNA aneuploidy. S-phase fraction was low (S + G2 <25%), except for sample 18 (60%).

Growth characteristics of untreated multicellular tumour spheroids

Non-irradiated MTS growth curves (Figure 1) were characterised by an initial growth followed by a plateau and in some cases by a decrease. Only sample 4 exhibited a decrease after 5 days, whereas the other samples exhibited a decrease only after 7 days or more. In one case (sample 14), the growth of MTS was linear ($R = 0.965$) for 10 days.

The doubling time during the initial growth varied from 1.0 to 8.5 days, with a mean (±SD) of 3.26 (±0.9) days (Table 3). For every individual tumour, the population of MTS was heterogeneous in size with mean diameters (± SE) ranging from 158 (±45) to 227 (±94) μm, with a mean of 198 (± 7.7) μm (Table 3). Only cell clusters with a diameter above 100 μm were followed-up.

Irradiated multicellular tumour spheroid control curves

Dose–effect curves representing the relative number of cured MTS 7 days after the irradiation versus irradiation dose were plotted (Figure 2). The dose required to reduce the number of spheroids by 50% compared with the control (SCD_{50}) varied from 1.9 to 15.7 Gy, with a mean (± SD) of 7.0 Gy (±1.8) (Table 3).

Radiation dose–response curves

The dose required to reduce the size of the MTS by 50% (ID_{50}), 7 days after irradiation was graphically estimated from the 6 MTS samples analysed (Figure 3) and ID_{50} varied from

Table 1. Samples characteristics: histopathology and origin, spheroids formation and DNA ploidy

Sample	Age (years)	Histopathology and origin of ovarian tumour	Spheroids formation	DNA ploidy (%)
1*	80	AF: ovarian carcinoma	Yes	—
2	72	PE: papillary adenocarcinoma	Yes	—
3	45	AF: moderately differentiated mucinous adenocarcinoma	No	Aneuploid (63.0%)
4	38	ST: endometrioid adenocarcinoma	Yes	Diploid
5	66	AF: mixed tumour, mullerian carcinosarcoma	No	Aneuploid (88.5%)
6	62	AF: serous surface papillary carcinoma	No	Diploid
7	54	AF: moderately differentiated serous adenocarcinoma	No	Aneuploid (13.5%)
8	49	AF: moderately differentiated adenocarcinoma	Yes	Diploid
9	59	AF: serous adenocarcinoma	Yes	Diploid
10	50	ST: mucinous papillary cystadenocarcinoma	No	—
11	64	AF: cystadenocarcinoma	No	Aneuploid (79.0%)
12	89	ST: adenocarcinoma	Yes	Diploid
13	74	ST: serous cystadenocarcinoma	No	Diploid
14	50	ST: serous adenocarcinoma	Yes	Diploid
15	49	ST: mucinous cystadenocarcinoma	No	—
16	71	ST: serous adenocarcinoma	No	Aneuploid (57.0%)
17†	57	ST: endometrioid adenocarcinoma	No	Aneuploid (65.0%)
18	77	AF: serous papillary adenocarcinoma	Yes	Aneuploid (41.0%)

AF, ascitic fluid; PE, pleural effusion; ST, solid tumour.

*The patient had received chemotherapy and hormonal treatment (melphalan and prodasone).

†The patient had received chemotherapy (cyclophosphamide—cisplatin) and radiotherapy.

Table 2. Immunocytochemical and flow cytometry analysis of the samples which formed spheroids

	Immunocytochemical analysis Monoclonal antibodies tested			Flow cytometry analysis						
								Cell cycle‡		
Sample Nos	KL_1	Anti-Vm	OC125	KL_1*	Anti-Vm*	OC125*	DNA ploidy	%G1	%G2	%S
14	++	++	++	27%	33%	2%	2.05	76.8	7.7	16.3
11	++	+	—	15%	20%	0%	NT	NT	NT	NT
4	NT	NT	++	NT	NT	NT	2.00	80.2	8.5	11.3
2	NT	NT	NT	80%	NT	0%	NT	NT	NT	NT
8	NT	NT	NT	2%	5%	4%	2.05	73.8	13.3	12.9
9	++	++	++†	36%	55%	5%	2.05	81.2	5.3	13.5
12	++	++	++	63%	58%	34%	NT	NT	NT	NT
18	++	++	++	12%	64%	12%	1.3	39.7	22.9	37.3

*Percentage of labelled cells as compared with the total cell number; †some mesothelial cells were labelled by the OC125; ‡analysis performed in the cells before irradiation; NT, not tested.

0.56 to 9.15 Gy with a mean value of 2.8 Gy ± 2.9 (SD) (Table 3).

The residual volume of MTS at 2 Gy at day 7 (RSV_2) studied in 8 samples varied from 10 to 88% with a mean value (±SD) of 44% (±25.5).

In comparing the RSV_2 and the S-phase fraction, sample 18 was found to be less radiosensitive than the other samples with a RSV_2 value of 88% whereas the S-phase fraction was higher (37.3%). Moreover, the SCD_{50} confirmed this relative radioresistance since 15.7 Gy irradiation dose was needed to destroy 50% of the MTS. A high RSV_2 value was also found for sample 9 (76%) despite a lower S-phase fraction (13%) but also a high SCD_{50} value approximating 10 Gy.

A significant correlation ($P = 0.02$) was found between SCD_{50} and RSV_2, which means that this parameter also characterises the radiosensitivity of the microtumours. ID_{50} was correlated to SCD_{50} ($P = 0.01$) and to RSV_2 ($P = 0.0009$) which means that the rate of tumour regression is related to the cellular kinetics, the dose delivered and the radiosensitivity. However, no correlation was found between ID_{50} and the doubling time. Neither SCD_{50}, ID_{50} nor RSV_2 were correlated to DNA ploidy.

DISCUSSION

Eight of the 18 (44%) tumour specimens formed MTS, probably because the stem cell fraction in human tumours is variable and usually small (<1%) [8]. However, if mucinous adenocarcinomas, which never formed MTS, were excluded the success rate was 53%. This might be improved further by using inactivated HeLa 'feeder' cells where the plating efficiency and the MTS formation from this hybrid spheroid assay [23] was increased.

Some discrepancies were found in the immunological characterisation using KL_1 [24] and antivimentin [25] antibodies. The ovarian adenocarcinoma, tumour of epithelial type, should have expressed only KL_1. However, some concomitant immunolabelling by antivimentin was found that may be explained by the fact that ovarian tumours originated from germinal ovarian epithelium of coelomic origin, i.e. of mesenchymal origin [26]. In case of differentiation, some tumour cells could co-express the antigenic structures recognised by the two monoclonal antibodies. The monoclonal antibody OC125 [27] was then used in order to distinguish epithelial cells from mesothelial cells. This antibody is classically specific of ovarian tumour epithelial cells [28], and it proved useful, despite some controversy [29–31], for the clinical diagnosis and the follow-up of patients with ovarian cancer. We observed [32], as have others [33], that mesothelial and granulocytic cells are labelled by OC125. Other monoclonal antibodies ($BerEP_4$, AuA_1) were tested and found more specific for epithelial structures [34].

The MTS growth curves we obtained were consistent with those previously observed by Schwachöfer [13] and Sutherland [8], who showed that the increase in size of MTS induced a progressive decrease in the number of proliferative cells and an increase in the number of quiescent non proliferative cells due to the limited diffusion of oxygen and other nutrients such as glucose. The mean doubling time was 3.26 days, which was close to the 2.65 days measured by Slotman and colleagues [35] on four ovarian cells of human origin. Furthermore, Sutherland [8] confirmed that the MTS grown from established tumour cell lines or directly from primary tumour specimens showed growth kinetics similar to those of tumours *in vivo*.

With regard to the radiosensitivity of ovarian MTS, while SF2 and linear quadratic model parameters (α,β) were used for bidimensional models, SCD_{50} was proposed by several authors [3, 22] as the standard radiosensitivity parameter for MTS. The values obtained in our study (1.9–15.7 Gy for MTS ranging from 158 (±45) to 227 μm (±94) in diameter) were in agreement with those reported in other models: 5.9–11 Gy for 150–400 μm MTS obtained from soft tissue tumour cell lines [3]; 5.6–15.1 Gy for 250 μm MTS from melanoma [36]; and 3.3–6.2 Gy for smaller MTS (<100 μm) from melanoma [37]. Similar relatively wide ranges of radiosensitivity were also reported when primary cell culture originating from tumour of the same histological type were evaluated [8] for radiosensitivity.

Contrary to Hill and colleagues [38], who showed that a high value of SCD_{50} was linked to a high fraction of proliferative cells, we only observed one case (sample 18) where these two parameters were linked. In addition, no correlation was observed between the DNA content and the radiosensitivity, as already reported by Allalunis-Turner and colleagues [39].

A correlation was found between SCD_{50} and ID_{50} confirming that the tumour response is correlated with local control. The local control, determined by SCD_{50}, depends essentially on intrinsic cellular radiosensitivity [14] which is defined by the survival ratio at 2 Gy (SF_2). The few studies concerning the radiosensitivity of MTS from ovarian tumour samples were carried out using clonogenic assays. Slotman and colleagues [35] reported SF_2 values varying from 0.07 to 0.38 in four ovarian cell lines, while Rofstad and associates [40] reported ranges between 0.14 and 0.50 in primary culture from ovarian biopsies.

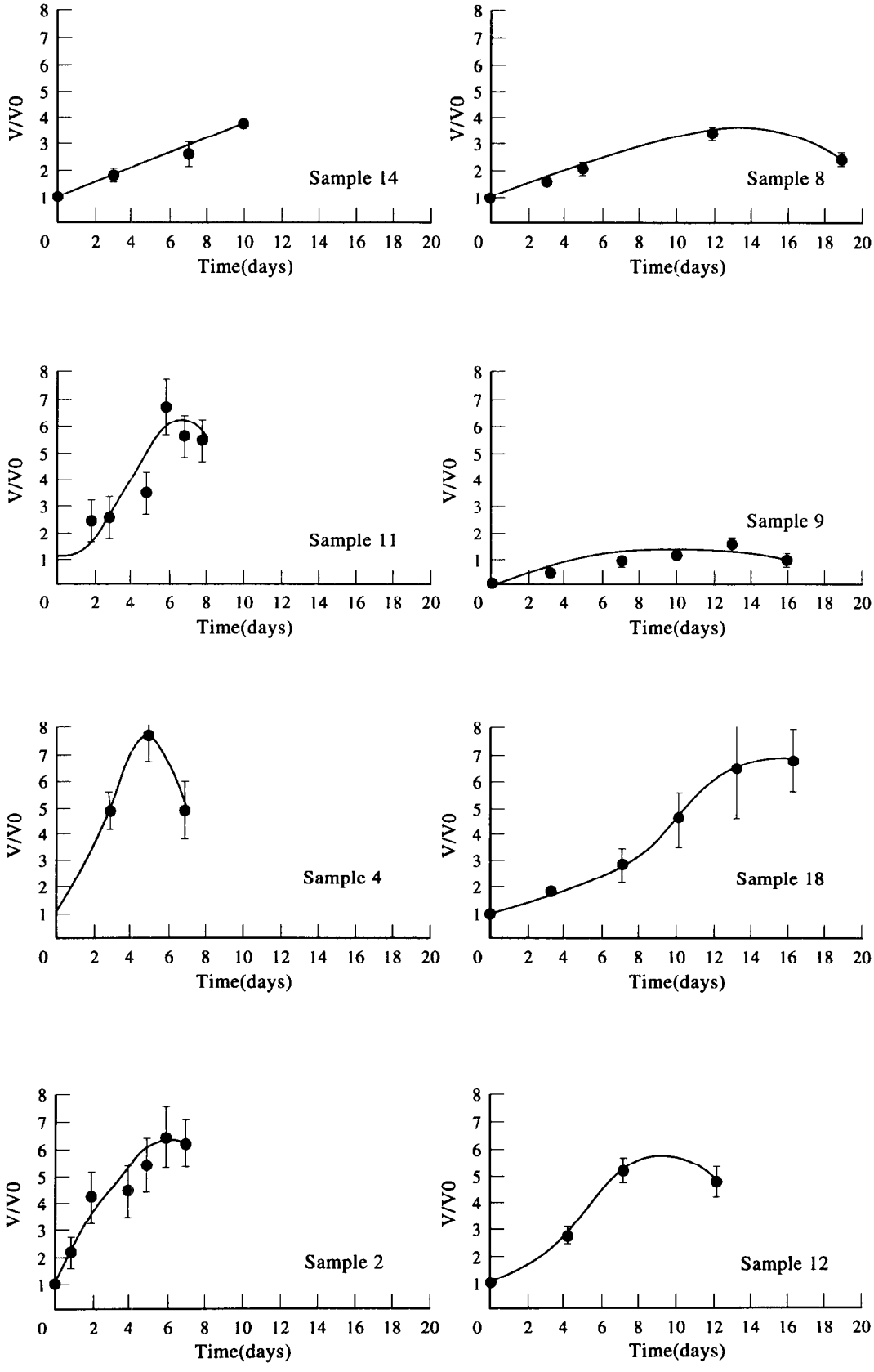

Figure 1. Growth curves of untreated spheroids obtained from each tumour. Each point was the mean volume of six different MTS. (Bars: Standard deviations.)

Table 3. Irradiation effects: radiosensitivity parameters calculated from the spheroids obtained from 8 tumours

Sample Nos	Spheroid diameter (μm) (mean ± SE)	RSV2* (%)	Doubling time (days) (mean ± SEM)	ID50† (Gy)	SCD50‡ (Gy)
14	200 (±112)	15	2.56 (±1.36)	0.56	4.70
11	192 (±83)	46	4.20 (±2.2)	1.78	2.30
4	193 (±83)	10	1.00 (±0.5)	0.64	2.90
2	195 (±76)	31	1.15 (±0.75)	0.94	1.90
8	158 (±45)	47	3.50 (±0.75)	1.80	10.00
9	227 (±94)	76	8.50 (±2.12)	6.32	9.85
12	214 (±75)	37	2.50 (±0.60)	1.19	9.30
18	210 (±71)	88	2.70 (±0.45)	9.15	15.72
Mean (±SD)	198 (±7.7)	44.0 (±25.5)	3.26 (±0.9)	2.80 (±2.9)	7.00 (±1.8)

SE, standard error; SD, standard deviation; *RSV2, residual spheroid volume at 2 Gy; †ID50, dose necessary to reduce the spheroid volume to 50%; ‡SCD50, dose necessary to reduce the spheroid number by 50% as compared with untreated control.

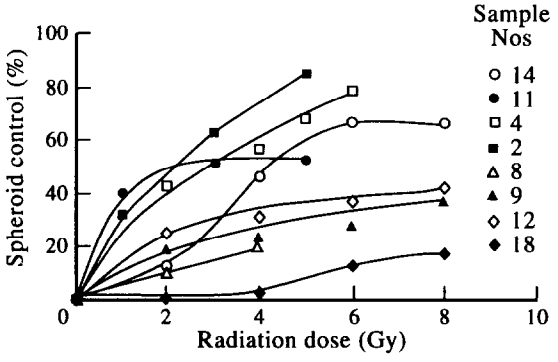

Figure 2. Multicellular tumour spheroids control curves of each tumour 7 days after irradiation. From these curves, the dose required to reduce the number of MTS to 50% of control (SCD50) was calculated mathematically.

In the present study, the relatively low number of MTS obtained did not allow us to determine SF_2, and this was the reason why the RSV_2 parameter was introduced to define the tumour regression 7 days after a 2 Gy irradiation. The results showed that the RSV_2 parameter was correlated with SCD_{50}.

The reproducibility of the assay was not indicated since the results were obtained from independent experiments performed with cells obtained from different ovarian surgical specimens whose sizes were too small to allow any repetitive experiment. Identical experiments were performed in seven ovarian human tumour cell lines, and relatively good reproducibility was obtained with standard deviations ranging from 4 to 23% for all radiosensitivity parameters (data not published). No correlation analysis of the radiosensitivity of MTS with clinical radioresponsiveness was possible, although, among the 18 patients, 6 were irradiated. Unfortunately, no patients whose specimen formed MTS was submitted to radiotherapy. However, Rofstad and colleagues [41] have indicated that the radiosensitivity of spheroids initiated directly from human melanoma surgical specimens reflected the clinical radioresponsiveness of the parent tumours.

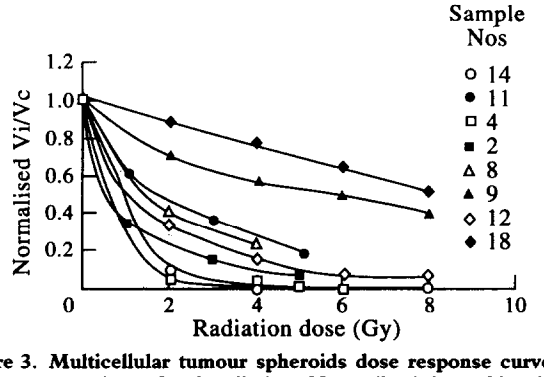

Figure 3. Multicellular tumour spheroids dose response curves of each tumour 7 days after irradiation. Normalised data of irradiated Volume (Vi)/control volume (Vc). Each point was the mean volume of six different MTS.

As a general conclusion, spheroids can be considered as *in vitro* models of tumour microregions reflecting the particular environmental conditions of cancer cells located at different distances from vessels [12]. This model appears to be an excellent tumour model for ovarian cancers, simulating the conditions found in the peritoneum, where nodular structures similar to spheroids are found at advanced stages of growth [12]. This method proved useful for studying the radiosensitivity of ovarian carcinomas by the determination of SCD_{50}. Because of the great variability of the clinical radiosensitivity of ovarian tumours, some parameters, which could help predict therapeutic outcome, should be determined for each patient, if radiotherapy is proposed as part of the therapeutic protocol. The present results, obtained using a three-dimensional *in vitro* model, can be considered an advancement in the prediction of the response of ovarian tumours to radiotherapy.

An improvement of individual *in vitro* radiosensitivity assays might be found in the three-dimensional histoculture on collagen sponge gels, allowing fresh surgical specimens to maintain their cell-to-cell contact and three-dimensional native tissue architecture in culture. This technique was recently developed and adapted by Hoffman and associates [5] for use in predictive assays of chemotherapeutic response and evaluation of new cancer drugs. Although this tumour model has not been studied for radiosensitivity testing, it should prove very interesting since collagen–sponge–gel histocultures approximate the *in vivo* state in which the tissues are growing in three dimensions, and which contain not only the tumour cells, but also stromal cells, such as fibroblasts and lymphocytes, as well as extracellular matrix components, all maintained in native architecture [42], thus differing from MTS which are only composed of tumour cells [43].

1. Slotman BJ, Rao BR. Ovarian cancer (Review). Etiology, diagnosis, prognosis, surgery, radiotherapy, chemotherapy and endocrine therapy. *Anticancer Res* 1988, **8**, 417–434.
2. Dembo AJ. Radiotherapeutic management of ovarian cancer. *Semin Oncol* 1984, **11**, 238–250.
3. Stuschke M, Budach V, Klaes W, Sack H. Radiosensitivity, repair capacity and stem cell fraction in human soft tissue tumors: an *in vitro* study using multicellular spheroids and the colony assay. *Int J Radiat Oncol Biol Phys* 1992, **23**, 69–80.
4. Freeman AE, Hoffman RM. *In vivo*-like growth of human tumors *in vitro*. *Proc Natl Acad Sci* 1986, **83**, 2694–2698.
5. Furukawa T, Kubota T, Watanabe M, *et al.* High *in vitro–in vivo* correlation of drug response using sponge-gel-supported three-dimensional histoculture and the MTT end point. *Int J Cancer* 1992, **51**, 489–498.
6. Hoffman RM. To do tissue culture in two or three dimensions? That is the question. *Stem Cell* 1993, **11**, 105–111.
7. Sutherland RM, McCredie JA, Inch WR. Multicellular spheroids: a new model target for *in vitro* studies of immunity to solid tumor allografts. *J Natl Cancer Inst* 1971, **46**, 113–120.
8. Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* 1988, **240**, 177–184.
9. Rofstad EK. Growth and radiosensitivity of malignant melanoma multicellular spheroids initiated directly from surgical specimens of tumours in man. *Br J Cancer* 1986, **54**, 569–578.
10. Tannock IF, Kopelyan I. Variation of pO2 in the growth medium of spheroids: interaction with glucose to influence spheroid growth and necrosis. *Br J Cancer* 1986, **53**, 823–827.
11. Twentyman PR. Response to chemotherapy of EMT6 spheroids as measured by growth delay and cell survival. *Br J Cancer* 1980, **42**, 297–304.
12. Mueller-Klieser W. Multicellular spheroids. A review on cellular aggregates in cancer research. *J Cancer Res Clin Oncol* 1987, **113**, 113–122.
13. Schwachöfer JHM. Multicellular tumor spheroids in radiotherapy research (Review). *Anticancer Res* 1990, **10**, 963–970.
14. Schwachöfer JHM, Crooijmans RPMA, Hoogenhout J, Kal HB. The influence of once or twice daily irradiation regimens on growth of squamous cell carcinoma spheroids of different diameters. *Anticancer Res* 1991, **11**, 1365–1368.
15. Sutherland RM, Durand RE. Radiation response of multicell spheroids: an *in vitro* model. *Curr Top Radiat Res Q* 1976, **11**, 87–139.
16. Frank C, Weber KJ, Fritz P, Flentje M. Increased dose-rate effect in V79-multicellular aggregates (spheroids). Relation to initial DNA lesions and repair. *Radiother Oncol* 1993, **26**, 264–270.
17. Weber B, Matrat M, Marchal S, Marchal C, Metz R. Usefulness of tumoral cells culture in agar. *Ann méd Nance Est* 1983, **22**, 513–516.
18. Ganter P, Jolles G. In Gautiers-Villars eds. *Histochimie Normale et Pathologique*, Vol. 2, Paris. 1970, 925–1904.
19. Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. *J Histochem Cytochem* 1984, **29**, 1196–1204.
20. Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1983, **3**, 323–327.
21. Yuhas JM, Li AP, Martinez AO, Landman AJ. A simplified method for production and growth of multicellular tumor spheroids. *Cancer Res* 1977, **37**, 3639–3643.
22. Schwachöfer JHM, Crooijmans RPMA, Hoogenhout J, Jerusalem CR, Kal HB, Theeuwes AGM. Radiosensitivity of different human tumor cell lines grown as multicellular spheroids derived from growth curves and survival data. *Int J Radiat Oncol Biol Phys* 1989, **17**, 1015–1020.
23. Lange CS, Djordjevic B, Brock WA. The hybrid spheroid clonogenic assay for the intrinsic radio- and chemo-sensitivities of human tumors. *Int Radiat Oncol Biol Phys* 1992, **24**, 511–517.
24. Viac J, Reano A, Brochier J, Staquet MJ, Thivolet J. Reactivity pattern of a monoclonal antikeratin antibody (KL_1). *J Invest Dermatol* 1983, **81**, 351–354.
25. Azumi N, Battifora H. The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. A comprehensive immunohistochemical study on formalin- and alcohol-fixed tumors. *Am J Clin Pathol* 1987, **88**, 286–296.
26. Van Niekerk CC, Ramaekers FCS, Hanselaar AGJM, Aldeweireldt J, Poels LG. Changes in expression of differentiation markers between normal ovarian cells and derived tumors. *Am J Pathol* 1993, **142**, 157–177.
27. Bast RC, Feeney M, Lazarus M, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981, **68**, 1331–1337.
28. Klug TL, Bast RC, Niloff JM, Knapp RC, Zurawski VR. Monoclonal antibody immunoradiometric assay for an antigenic determinant (CA125) associated with human epithelial ovarian carcinoma. *Cancer Res* 1984, **44**, 1048–1053.
29. Kuesel AC, Kroft T, Préfontaine M, Smith ICP. Lipoprotein (a) and CA125 levels in the plasma of patients with benign and malignant ovarian disease. *Int J Cancer* 1992, **52**, 341–346.
30. Pinto MH, Bernstein LH, Rudolph RA, Brogan DA, Rosman M. Diagnostic efficiency of carcinoembryonic antigen and CA125 in the cytological evaluation of effusions. *Arch Pathol Lab Med* 1992, **116**, 626–631.
31. Van Nagell JR, DePriest PD, Gallion HH, Pavlik EJ. Ovarian cancer screening. *Cancer* 1993, **71**, 1523–1528.
32. Griffon G, Merlin JL, Parache RM, Leroux-Broussier A, Marchal S, Marchal C. Radiosensitivity of spheroids obtained from human ovarian tumors: preliminary results. In Dittrich CH, Arps H, Aapro MS, eds. *Drugs, Cells and Cancer, EORTC Preclinical Therapeutic Models Group*, 1992 Annual Report, Welley, Vienna, 1992, 42–47.
33. Kabawat SE, Bast RC, Bhan AK, Welch WR, Knapp RC, Colvin RB. Tissue distribution of a coelomic epithelium-related antigen recognized by the monoclonal antibody OC125. *Int J Gynecol Pathol* 1983, **2**, 275–285.
34. Griffon G, Marchal C, Merlin JL, Parache RM, Marchal S. Approche expérimentale de la radiosensibilité des adénocarcinomas ovariens. *Bull Cancer* 1993, **80**, 456.
35. Slotman BJ, Karim ABMF, Rao BR. Ovarian cancer: radiation sensitivity *in vitro*. *Radiother Oncol* 1990, **19**, 323–327.
36. Kuwashima Y, Majima V, Okada S. Cure, cell killing, growth delay and fragmentation of X-irradiated human melanoma HMV-I multicellular spheroids. *Int J Radiat Biol* 1988, **54**, 91–104.
37. Rofstad EK, Wahl A, Davies C, Brustad T. Growth characteristics of human melanoma multicellular spheroids in liquid overlay culture: comparisons with the parent tumour xenografts. *Cell Tissue Kinet* 1986, **19**, 205–216.
38. Hill RP, Milas L. The proportion of stem cells in murine tumors. *Int J Radiat Oncol Biol Phys* 1989, **16**, 513–518.
39. Allalunis-Turner MJ, Pearcey RG, Barron GM, Buryn DA, Babiak JC, Honoré LH. Inherent radiosensitivity testing of tumor biopsies obtained from patients with carcinoma of the cervix or endometrium. *Radiother Oncol* 1991, **22**, 201–205.
40. Rofstad EK, Wahl A, Brustad T. Radiation sensitivity *in vitro* of cells isolated from human tumor surgical specimens. *Cancer Res* 1987, **47**, 106–110.
41. Rofstad EK, Wahl A, Brustad T. Radiation response of multicellular spheroids initiated from five human melanoma xenograft lines. Relationships to the radioresponsiveness *in vivo*. *Br J Radiol* 1986, **59**, 1023–1029.
42. Hoffman RM. Histoculture and the immunodeficient mouse come to the cancer clinic: rational approaches to individualizing cancer therapy and new drug evaluation (Review). *Int J Oncol* 1992, **1**, 467–474.
43. Twentyman PR. Exclusion of host cells during spheroid formation from disaggregated solid tumours. *Br J Cancer* 1983, **47**, 541–543.

Acknowledgements—The authors would like to thank Mrs O. Vigne and Mrs A. Leclerc for their help in the immunocytochemical analysis, Drs P. Aletti and A. Noël for performing the irradiation, Dr C. Barlier-Pagel for her kind gift of some tumour specimens, Dr A. Courdi for reading and discussing this paper and Ms D. Chanel for her secretarial assistance.

This work was performed within the framework of the EORTC-Preclinical Therapeutic Model Group and was supported by the Ligue Nationale Contre le Cancer and its committee of Meurthe et Moselle.