J. Pharm. Pharmacol. 1998, 50: 285–290
Received June 3, 1997
Accepted November 17, 1997

Study of the Effects of Dispase in the Chemotherapy of Multicellular Tumour Spheroids of Small-cell Lung Carcinoma in Man

HIDEHITO MATSUOKA, MASAYOSHI OKADA, NOBORU ISHII AND SAKAN MAEDA*

*Department of Surgery Division II and *The Second Department of Pathology, Kobe University School of Medicine, Kobe, Japan*

Abstract

Multicellular tumour spheroids (MTS), diameter 650 μm, from PC-6, SBC-1 and NCL-H60 small-cell lung carcinoma cell-lines in man were prepared by the liquid overlay culture method and used to study the influence of treatment with dispase (bacterial neutral protease from *Bacillus polymyxa*, 1000 units mL^{-1}) on the effectiveness of carboplatin, as determined by colony-forming assay.

When carboplatin alone was used on monolayers the curve of survival fraction against concentration was exponential in shape, indicating that the drug was active against the monolayer. When MTS were treated with medium concentrations (10^{-5} and 10^{-4} M) of carboplatin alone the survival fraction–concentration curve showed that the effectiveness of the treatment was less than that against the monolayer. On treatment of MTS with carboplatin and dispase the survival fraction–concentration curve was similar to that obtained for the monolayer and the survival fraction of the core of the MTS was also less than when carboplatin alone was used.

These results imply that dispase dissolves the intercellular matrix of the MTS enabling enhanced infiltration of carboplatin into the core of the MTS. Dispase thus indirectly increases the effectiveness of carboplatin.

Small-cell lung carcinoma is often treated by chemotherapy rather than surgery even at early stages of the disease. The three-dimensional structure of the tumour can prevent penetration of the anti-cancer drug into the core of the tumour. Multicellular tumour spheroids have been used as three-dimensional in-vitro tumour models in many studies. In this paper we describe how dispase, a bacterial neutral protease from *Bacillus polymyxa*, which can dissolve the intercellular matrix of multicellular tumour spheroids (MTS) from human lung small-cell carcinoma cell-lines, increased penetration of carboplatin into the core and as a result indirectly enhanced the effects of the drug.

Materials and Methods

Cell-lines

Experiments were performed on three cell-lines from human lung small-cell carcinoma—PC-6, SBC-1 and NCL-H60. Monolayer cultures were maintained in RPMI-1640 complete culture medium (Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (Gibco), benzylpenicillin potassium (50 units mL^{-1}), streptomycin sulphate (50 μg mL^{-1}) and fluconazole (10 μg mL^{-1}). The cell-lines were grown in an atmosphere of 100% relative humidity, 95% air–5% CO_2 at 37°C in our laboratory.

MTS production

MTS were produced by the liquid overlay culture method described by Yuhas et al (1977). Viable cells of three cell-lines (10^5 mL^{-1}) in complete culture medium were placed in 60-mm plastic Petri dishes (Falcon, Cockysville, MD), previously coated with complete culture medium containing 0·5% Noble agar (Difco Laboratories, Detroit, MI), and incubated in an atmosphere of 100% relative humidity, 95% air–5% CO_2 at 37°C. Aggregated cells were transferred into fresh complete culture medium on new agar-coated plates once a week by use of 1-mL pipettes (Falcon). After three weeks

Correspondence: H. Matsuoka, Department of Surgery Division II, Kobe University School of Medicine, Kusunokicho 7-5-2, Chuo-ku, Kobe 650, Hyogo, Japan.
E-Mail mahide@sanynet.ne.jp

aggregated cells became MTS with a diameter of approximately 650 μm, the maximum size of MTS with viable core cells. These were used for subsequent studies.

Monolayer studies
When the three cell-lines were in exponential growth phase as monolayers, they were treated with carboplatin diluted to different concentrations (10^{-6}, 10^{-5}, 10^{-4} or 10^{-3} M) in complete culture medium and incubated for 1 h at 37°C in 95% air–5% CO_2. After incubation, a single-cell suspension was obtained by trypsinization with 0·2% trypsin containing 0·01% EDTA. After inactivation of the trypsin by addition of FBS, the cells were gently washed twice with the RPMI-1640 medium and resuspended in complete culture medium with 0·3% Noble agar. The number of cells was adjusted to 10^4 mL^{-1}. For colony assay this suspension (1 mL) was placed in 35-mm Petri dishes (Falcon) previously coated with 1 mL complete culture medium containing 0·5% Noble agar and incubated in an atmosphere of 100% relative humidity and 95% air–5% CO_2 at 37°C. After two weeks colonies of 50 cells were counted. As a control, cells from a monolayer which had not been treated with carboplatin were adjusted to 10^4 cells mL^{-1} and subjected to the same colony assay under the same atmospheric conditions. The concentration–response curve was drawn by plotting the number of colonies as a percentage of control against each drug concentration. Each experiment was performed thirty times and each measurement was repeated at least three times.

MTS studies
MTS of approximate diameter 650 μm were treated with carboplatin diluted with complete culture medium to the concentration used for monolayer studies and incubated for 1 h at 37°C in 95% air–5% CO_2. After incubation a single-cell suspension was obtained by trypsinization with 0·2% trypsin containing 0·01% EDTA. After inactivation of the trypsin by addition of FBS the cells were gently washed twice with RPMI-1640 medium and resuspended in complete culture medium containing 0·3% Noble agar. The cell numbers were adjusted to 10^4 mL^{-1}. For colony assay this suspension (1 mL) was placed in 35-mm Petri dishes (Falcon), previously coated with 1 mL complete culture medium containing 0·5% Noble agar, and incubated in an atmosphere of 100% relative humidity and 95% air–5% CO_2 at 37°C. After two weeks colonies of 50 cells were counted. As a control, cells from multicellular tumour spheroids which had not been treated with carboplatin were adjusted to 10^4 cells mL^{-1} and subjected to the same colony assay under the same atmospheric conditions. The concentration–response curve was drawn by plotting the number of colonies as a percentage of control against each drug concentration. Each experiment was performed thirty times and each measurement was repeated at least three times.

Effect of dispase alone on monolayers and on MTS
Three cell-lines, as monolayers and as 650 μm diameter MTS, were treated with 100, 500 or 1000 units mL^{-1} dispase (Godo Shusei, Matsudo, Chiba) and incubated in complete culture medium for 1 h at 37°C in 95% air–5% CO_2. After incubation, a single-cell suspension was obtained by the same trypsinization technique as used for previous monolayer and MTS studies. Using this single-cell suspension, the same colony assay was performed. Cells from monolayers and from MTS which had not been treated with dispase were used as controls. Each experiment was performed thirty times and each measurement was repeated at least three times.

Effect of combined dispase and carboplatin on MTS
MTS, 650 μm diameter, were treated simultaneously with carboplatin and dispase and incubated for 1 h at 37°C in 95% air–5% CO_2 in complete culture medium. The carboplatin concentration was the same as that used for the previous MTS studies. After incubation, a single-cell suspension was obtained by trypsinization and the same colony assay was performed. Cells from MTS which had not been treated with carboplatin or dispase were used as controls. Each experiment was performed thirty times and each measurement was repeated at least three times.

Effect of combined dispase and carboplatin on MTS cores
After the same 1-h treatment with carboplatin and dispase as in the previous study, the outer layers of the MTS were removed by use of 0·2% trypsin containing 0·01% EDTA. The trypsin was then inactivated by addition of FBS and the 150–200-μm-depth cell layer from the surface was dissolved while under microscopic observation. The residual cores of the MTS were then picked up microscopically by use of a micro-pipette and the colony assay perviously performed in the complete MTS study was performed on these MTS cores. Cores from MTS which had not been treated with carboplatin or dispase as controls. Each experiment was performed thirty times and each measurement was repeated at least three times.

Statistical analysis
Values are expressed as means ± s.e.m. All data were analysed by the two-tailed Student's *t*-test. *P* values < 0·05, compared with controls, were taken as indicative of statistical significance.

Results

MTS with a diameter of 650 μm were obtained in three weeks (Figure 1). The number of colonies formed in the controls were 163·4 ± 12·2, 132·2 ± 18·4 and 118·7 ± 11·6 for cell-lines PC-6, SBC-1 and NCL-H60, respectively; these values were used for subsequent calculation of survival fraction.

Effect of dispase against small-cell carcinoma cell-lines both as monolayers and as MTS
Dispase alone did not influence the survival fraction for the three cell-lines whether as monolayers or as MTS.

For PC-6 monolayers the survival fraction was 95 ± 9% for dispase concentrations of 100 and 500 units mL^{-1} and 95 ± 10% for a dispase concentration of 1000 units mL^{-1}. For PC-6 MTS the survival fraction was 92 ± 3% for a dispase concentration of 100 units mL^{-1} and 92 ± 5% for dispase concentrations of 500 and 1000 units mL^{-1}.

For SBC-1 monolayers the survival fraction was 95 ± 8% for dispase concentrations of 100 and 500 units mL^{-1} and 94 ± 8% for a dispase concentration of 1000 units mL^{-1}. For SBC-1 MTS the survival fraction was 92 ± 3% for a dispase concentration of 100 units mL^{-1} and 91 ± 7% for dispase concentrations of 500 and 1000 units mL^{-1}.

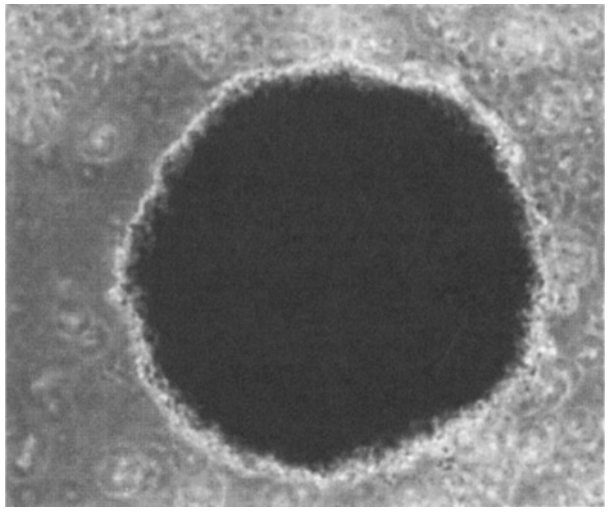

Figure 1. Multicellular tumour spheroid (PC-6, three weeks, 650 μm).

For NCL-H60 monolayers the survival fraction was 95 ± 8% for dispase concentrations of 100 and 500 units mL^{-1} and 94 ± 8% for a dispase concentration of 1000 units mL^{-1}. For NCL-H60 MTS the survival fraction was 92 ± 4% for dispase concentrations of 100 and 500 units mL^{-1} and 91 ± 5% for a dispase concentration of 1000 units mL^{-1}.

Effect of dispase on the effectiveness of carboplatin against both monolayers and MTS (Figure 2)
The survival of PC-6 monolayers was reduced exponentially by carboplatin alone whereas for carboplatin concentrations of 10^{-5} and 10^{-4} M the survival fractions for PC-6 MTS were significantly higher (80·6 ± 6·9% and 51·1 ± 9·5%, respectively). Treatment with both dispase and carboplatin did not affect the concentration–survival curve for monolayers; for MTS, however, the survival fractions were significantly ($P < 0·05$) reduced to 71·2 ± 4·8% and 41·4 ± 2·9% for carboplatin concentrations of 10^{-5} and 10^{-4} M, respectively. Similarly, although survival of SBC-1 monolayers was reduced exponentially by carboplatin the survival fractions for MTS were significantly higher, 82·3 ± 3·8% and 49·1 ± 10·3% for carboplatin concentrations of 10^{-5} and 10^{-4} M respectively. Treatment with both dispase and carboplatin did not affect the concentration–survival curve for SBC-1 monolayers but the survival fraction for SBC-1 MTS was significantly ($P < 0·05$) reduced to 70·6 ± 9·2% and 41·9 ± 8·4% respectively for carboplatin concentrations of 10^{-5} and 10^{-4} M.

Survival of NCL-H60 monolayers was reduced exponentially by carboplatin but for NCL-H60 MTS the survival fraction was significantly higher – 78·3 ± 9·8% for carboplatin concentrations of 10^{-5} M. Treatment with both dispase and carboplatin significantly ($P < 0·05$) reduced the survival fraction for NCL-H60 MTS (to 68·2 ± 7·9% for a carboplatin concentration of 10^{-5} M).

Effect of dispase on the effectiveness of carboplatin against the core of MTS (Figure 3)
Combined use of dispase and carboplatin significantly ($P < 0·05$) reduced the survival of the cores of PC-6, SBC-1 and NCL-H60 MTS; for carboplatin concentrations of 10^{-5} and 10^{-4} M survival was reduced from 93·6 ± 7·6 to 80·9 ± 8·4 and from 79·2 ± 8·2 to 67·7 ± 5·9, respectively, for the PC-6 cell-line; from 90·5 ± 6·9 to 78·7 ± 9·9 and from 73·6 ± 11·1 to 55·4 ± 9·8, respectively, for the SBC-1 cell-line; and from 91·0 ± 7·7 to 79·5 ± 11·3 and from 72·9 ± 12·8 to 56·8 ± 10·4, respectively, for the NCL-H60 cell-line.

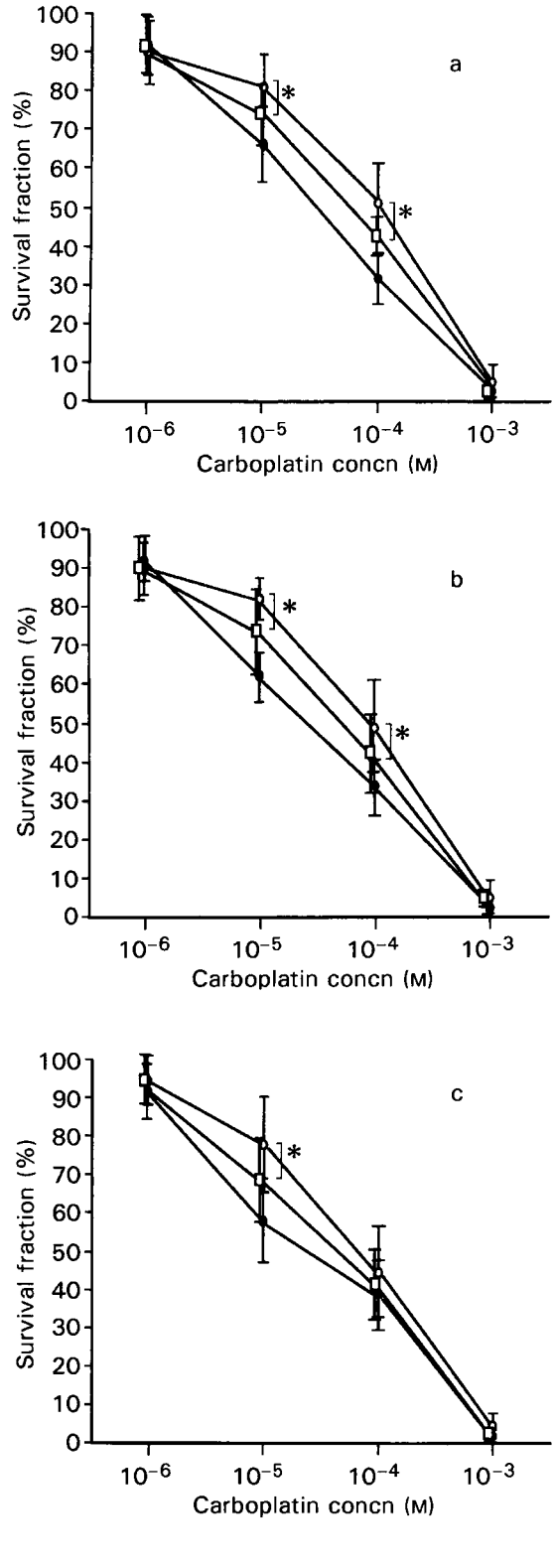

Figure 2. Curves of survival fraction against carboplatin concentration for (a) PC-6, (b) SBC-1 and (c) NCL-H60. ●, Monolayer treated with dispase and carboplatin; ○, MTS treated with carboplatin alone; □, MTS treated with dispase and carboplatin. Each point is the mean of results from 30 experiments; s.e.m. are indicated by the vertical bars. *$P < 0.05$, significantly different.

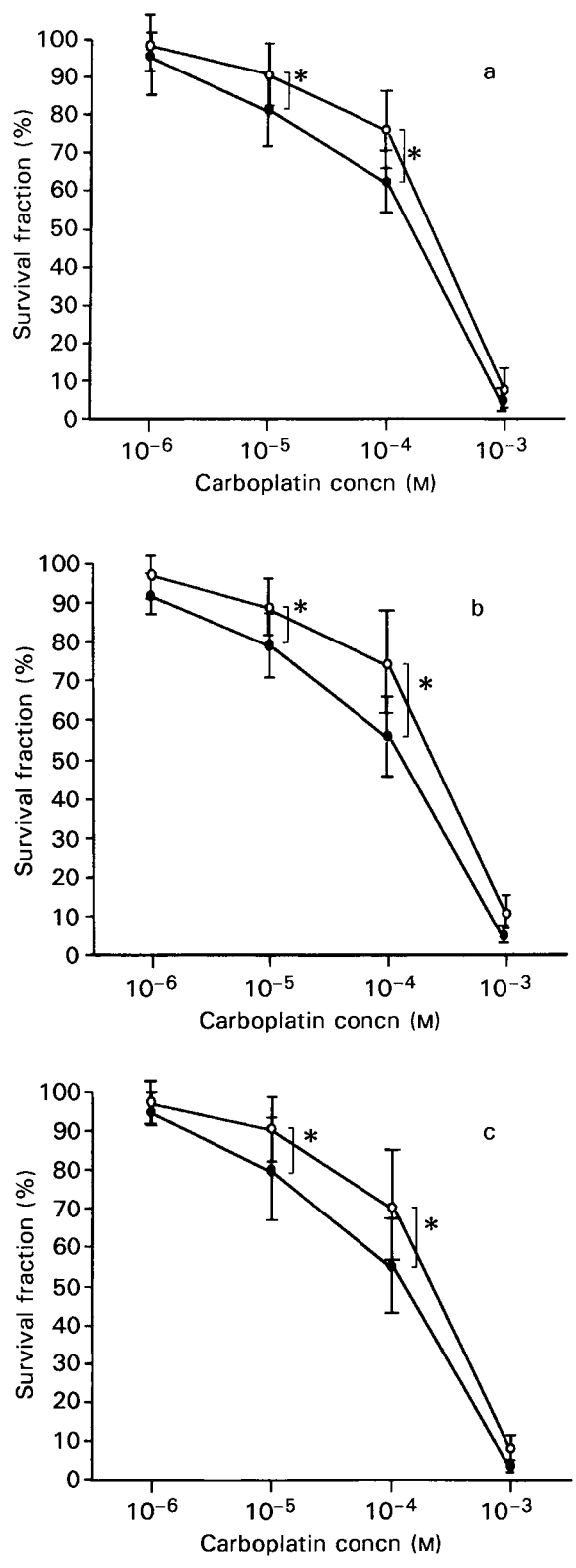

Figure 3. Curves of survival fraction against carboplatin concentration for (a) PC-6, (b) SBC-1 and (c) NCL-H60 MTS cores. ○, MTS treated with carboplatin alone; ●, MTS treated with dispase and carboplatin. Each point is the mean of results from 30 experiments; s.e.m. are indicated by the vertical bars. *$P < 0.05$, significantly different.

Discussion

MTS have characteristics of in-vivo tumours that monolayer cultures do not have. Originally MTS were maintained in semi-solid agar (McAllister et al 1967) or in spinner flasks (Inch et al 1970; Sutherland et al 1971). These methods had many limitations which affected the quality and quantity of the MTS produced. As a consequence of Sutherland's report that on agar-coated dishes cell-cell interaction was enhanced at the expense of cell-dish interactions, the liquid-overlay culture method was developed (Yuhas et al 1977) and MTS prepared by this method have been used for many studies of the effects of radiation or chemotherapy (Kuroki 1973; Franko & Sutherland 1979; West et al 1980; Inoue & Ohnuma 1989; Mashiyama et al 1989; Russell et al 1989; Schwachoefer et al 1989; Gaze et al 1992). Since the development of extracellular matrix analysis of glioma and thyroid cancer cells (Nederman et al 1984) attempts have been made to increase three-dimensional penetration into the core of MTS and thus enhance the performance of chemotherapy (Sasaki et al 1984). Kohno et al (1988) reported that pretreatment with cisplatin enhanced penetration of doxorubicin into the core of MTS. They also reported that hyaluronidase effected mild dissolution of the intercellular matrix and enhanced penetration of doxorubicin into the core of MTS. In the current study we used protease to dissolve the intercellular matrix of MTS and enhance penetration, in an attempt to increase effectiveness of the anticancer drug indirectly.

Dispase, first described by Matsumura et al (1975), is a bacterial neutral protease, molecular weight 35 900 (Irie 1976), released into culture media by *Bacillus polymyxa*. It is activated by Ca^{2+} and several other metal ions and inhibited by chelating agents such as EDTA. Dispase maintains its activity for days in a tissue culture medium supplemented with foetal bovine serum. As a result of these properties it is possible that dispase could become an important enhancer in future chemotherapy in-vivo. Dispase has been shown to have different actions on the intercellular matrix of different cell-lines (Nagata & Matsumura 1986). With fibroblast-like cells (BNK-21, CHO-K1, L-929, L6TG, V79) cell-dispersing capacity was good, with epithelial-like cells (8999C, FRSK, HeLa S3, JB6, JTC-16, MDCK, RK13, RLC-10(2), Vere) it was variable, and with melanoma cell B16 no cell-dispersing capability was apparent. No obvious dispersion has been reported for the three cell-lines of small-cell human carcinoma used in this study and they are regarded as hard to disperse.

When using such a protease in-vivo, cytotoxicity to normal cells is a serious problem. Nagata & Matsumura (1986) reported the cytotoxicity of dispase (500 units mL^{-1}) against several cell-lines (BHK21, CHO-K1, HeLa S3, JTC-16, L6TG, L-929, V79, 8999C). Long-term exposure to dispase resulted in cytotoxicity against all the cell-lines, but to different extents. Severe concentration-dependent damage was observed for CHO-K1, L-929, V79 and 8999C, and weak concentration-dependent damage for BHK21 and L6TG. Damage to HeLa S3 was low even when the dispase concentration was high and JTC-16 was severely damaged even by low concentrations of dispase. Short-term exposure of all cell-lines to a high concentration of dispase (500 units mL^{-1}, 40-min incubation) did not result in significant cytotoxicity.

In our study, no cytotoxicity was observed after 60-min incubation in 1000 units mL^{-1} dispase but use of dispase resulted in improved cell dispersion and improvement of the cytotoxicity of carboplatin. Reducing cytotoxicity against normal cells and selective administration to the tumour are required for in-vivo application.

References

Franko, A. J., Sutherland, R. M. (1979) Radiation survival cells from spheroids grown in different oxygen concentration. Radiation Res. 79: 454–467

Gaze, M. N., Mairs, R. J., Boyack, S. M. (1992) ^{131}I-*meta*-Iodobenzylguanidine therapy in neuroblastoma spheroids of different sizes. Br. J. Cancer 66: 1048–1052

Inch, W. R., McCredie, J. A., Sutherland, R. M. (1970) Growth of nuclear carcinomas in rodents compared with multicell spheroids in tissue culture. Growth 34: 271–282

Inoue, S., Ohnuma, T. (1989) Effects of physiological oxygen environment on drug-induced cell lethality multicellular tumour spheroids from human lung cancer. Selective Cancer Ther. 5: 13–22

Irie, Y. (1976) Neutral protease useful for animal tissue and cell culture. US Patent 3948725

Kohno, N., Ohnima, T., Kaneko, N. (1988) Interactions of doxorubicin and cisplatin in squamous carcinoma cells in culture. Br. J. Cancer 58: 330–334

Kuroki, T. (1973) Colony formation of mammalian cells on agar plates and its application to Lederberg's replica. Exp. Cell Res. 80: 55–62

McAllister, R. M., Reed, G., Huebner, R. J. (1967) Colonial growth in agar cells derived from adenovirus-induced hamster tumours. J. Natl Cancer Inst. 39: 43–53

Mashiyama, S., Katakura, R., Takahashi, K. (1989) Enhancement of the effect of X-irradiation against cultured human glioblastoma cells by pre-treatment with ACNU. Neurol. Med. Chir. 29: 1070–1077

Matsumura, T., Yamana, T., Hashizume, S. (1975) Tissue dispersion, cell harvest and fluid suspension culture by the use of bacterial neutral protease. Jpn J. Exp. Med. 45: 377–382

Nagata, M., Matsumura, T. (1986) Action of the bacterial neutral protease dispase on cultured cells and its application to fluid suspension culture with a review on biomedical

application of this protease. Jpn J. Exp. Med. 56: 297–307

Nederman, T., Norling, B., Glielius, B. (1984) Demonstration of extracellular matrix in multicellular tumour spheroids. Cancer Res. 44: 3090–3097

Russell, J., Adam, J., Wheldon, T. E. (1989) The relative effectiveness of analogues of cisplatin in the experimental chemotherapy of human non-small-cell lung cancer and neuroblastoma grown as multicellular spheroids. Cancer Chemother. Pharmacol. 23: 111–114

Sasaki, T., Yamamoto, M., Kuwahara, K. (1984) Lethal effect of bleomycin and peplomycin on HeLa cells in multicell tumour spheroids. Cancer Res. 44: 1374–1379

Schwachoefer, J. H. M., Crooijimans, R. P. M. A., Broers, J. L. V. (1989) Multicellular aggregates from human tumour cell-lines for radiation studies. Anticancer Res. 9: 441–448

Sutherland, R. M., McCredie, J. A., Inch, W. R. (1971) Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J. Natl Cancer Inst. 46: 113–120

West, G. W., Weichselbaum, R., Little, J. B. (1980) Limited penetration of methotrexate into human osteosarcoma spheroid as a proposed model for solid tumour resistance to adjuvant chemotherapy. Cancer Res. 40: 3665–3668

Yuhas, J. M., Li, A. P., Martinez, A. O. (1977) A simplified method for production and growth of multicellular tumour spheroids. Cancer Res. 37: 3639–3643