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Regulation of Endothelial CD44 Expression and Endothelium–Tumour Cell Interactions by Hepatocyte Growth Factor/Scatter Factor

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Cancer metastasis involves the passage of tumour cells into and out of blood or lymphatic circulatory systems and requires their interaction with the endothelial cells lining these vessels. Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional protein that enhances tumour cell motility and extracellular matrix invasion and has been implicated as a mediator of metastasis. In this study, we have investigated the effect of HGF/SF on tumour cell–endothelial cell interactions. A fluorescent tumour cell–endothelial cell attachment assay demonstrated that, following endothelial monolayer stimulation with HGF/SF, tumour cell attachment to endothelium is increased. Addition of anti-CD44 antibodies in this assay inhibited the effects of HGF/SF. Western blotting studies showed that HGF/SF increased expression of the adhesion molecule CD44 in endothelial cells. These results were confirmed by both immunohistochemical staining and a cell-surface adhesion molecule ELISA. These results suggest that HGF/SF plays a key role in the initial adhesion mechanism between tumour cells and endothelial cells via up-regulation of CD44.

Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional cytokine that stimulates profound motogenic responses in tumour cells, enhancing both their motile and invasive potential. Tumour cell motility and invasion are essential requirements for the metastatic spread of cancers and thus HGF/SF has been implicated in this pathophysiological process (1-4).

A key step in the metastatic spread of cancers is the arrest of circulating tumour cells in capillaries or lymphatic vessels near distant organs and their subsequent invasion through the circulatory vessel wall and extracellular matrix (4-6). In order for this to occur, the metastasising cells must be brought into close contact with the endothelial cell lining of the circulatory vessels thus allowing tumour cell-endothelial adherence. Once attached, they may extravasate into the surrounding extracellular matrix. A number of studies have demonstrated HGF/SF to be a potent stimulator of tumour cell invasion into extracellular matrix components such as collagen and fibronectin (for review, see refs 1 and 2) but its function on tumour cell-endothelial interactions are not widely reported.

Metastasising tumour cells share many properties with activated lymphocytes in that they both exhibit invasive behaviour and migration via reversible adhesive contacts and recent data suggests that common molecular mechanisms may be involved in these phenomena (7).

Here we report that HGF/SF up-regulates the expression of CD44 receptors on the surface of endothelial cells and enhances tumour cell-endothelial adhesions. These observations further implicate HGF/SF as a key mediator of metastatic tumour spread.

MATERIALS AND METHODS

Cell lines. A human vascular endothelial cell line, ECV304, together with a human colon cancer cell line, HT115, were obtained from the European Collection of Animal Cell Cultures (ECACC, Sailsbury, U.K.). The human breast cancer cell line, MCF7 was obtained from the American Tissue Type Collection (ATCC, Maryland, U.S.A). HT115 and MCF7 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma Ltd, Poole, Dorset, UK) supplemented with 10% foetal calf serum; ECV304 cell were cultured in M199 medium (GibcoBRL) containing 10% serum.

Materials. The fluorescent label, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) was purchased from Molecular Probes Inc. (Eugene, Oregon, USA) and stored as a 5mM stock solution (in DMSO) at −20°C. Human recombinant HGF/SF was a generous gift from Dr. T. Nakamura, Osaka, Japan. Anti CD44, E-selectin and VCAM-1 antibodies were obtained from R&D systems Ltd (Abingdon, Oxford, UK) together with an anti mouse peroxidase-conjugated IgG was from Amersham Ltd (Little Chalfont, Bucks, UK). A chemiluminescence detection kit was purchased from Insight Biotechnology (Wembley, Middlesex). All other materials were from Sigma Ltd unless otherwise stated.

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Tumour cell-endothelial cell attachment assay. Labelled tumour cells were seeded onto HGF/SF-treated (or plain medium as control) endothelial monolayers in 96-well plates at 5×10^4 cells/well. Tumour cells were co-cultured with the endothelial cells for 50 minutes following which the wells were washed twice with BSS. The plates were then read using a fluorescent plate reader (Denley, Sussex, UK) with λ (excitation) set at 540nm and λ (emission) set at 590nm. Results are shown as mean of three experiments ± S.E.M.

Fluorescent labelling of tumour cells. Tumour cells were first labelled with the fluorescent marker, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI), prior to their addition to endothelial cell-coated wells of a 96-well plate as described previously (8).

Cellular adhesion molecule ELISA. Expression of adhesion molecules on the surface of the endothelium was facilitated by an ELISA method based on that described by Rice *et al* (9). Equal numbers of ECV304 cells were seeded into the wells of a 96-well ELISA plate and allowed to form monolayers. Following stimulation with HGF/SF for between 30 minutes and 16 hours, the wells were washed and cells fixed with 100% methanol. Following blocking with 10% milk protein (in TBS), primary antibodies were added (0.2μg/ml in TBS containing 3% milk) and incubated at room temperature for 1 hour. Monolayers were then washed and the secondary antibody added (peroxidase-conjugated IgG, 1:5,000 in 3% milk solution). Colour was developed by the addition of 0.6mg/ml 1,2-phenylenediamine dihydrochloride (OPD) (+0.15% H_2O_2) for 15 minutes, after which the reaction was terminated with 3N H_2SO_4. Plates were then read in a Titerteck plate reader at λ=490nm.

Western blotting. Endothelial cell cultures were treated with HGF/SF (40ng/ml) for either 30 minutes, 1, 2, 4 or 8 hours. Cells were then pelleted at 1200 rpm prior to lysis in cell lysis buffer (50mM Tris, pH 7.8, 150mM NaCl, 1% Triton, 0.1% SDS, 0.02% NaN_3, 10mM NaF, PMSF 100μg/ml, Leupeptin 2μg/ml) at 4°C for 30 minutes. Cellular debris was removed by centrifugation at 12000 rpm following which sample protein concentration was determined. After addition of electrophoresis sample buffer (50mM Tris, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol) and boiling, equal amounts of protein were separated with SDS-PAGE and transferred onto an inert support by electro blotting. Immunoblots were probed with either anti-CD44, E-selectin or VCAM-1 antibodies (in TBS containing 3% milk protein and 0.1% Tween 20). Bound primary antibodies were detected by peroxidase-conjugated immunoglobulins and an enhanced chemiluminescence system. After developing the films, the antibody signals were quantitated by measuring band densities using scanning laser densitometry.

Immunohistochemical staining of cell-surface CD44. Endothelial cell monolayers were activated with HGF/SF (40ng/ml) for 4 hours then fixed with formalin. Following inhibition of endogenous peroxidases with H_2O_2 (0.3% in methanol) and blocking with milk solution (10% in BSS, + 0.1% Tween20), anti-CD44 antibodies were added and incubation carried out for 1 hour at room temperature. After extensive washing, bound CD44 was detected by means of a peroxidase-conjugated secondary antibody together with diamino benzidine (DAB, 180μg/ml in 0.025M Tris, pH7.4 containing 0.015% H_2O_2). Cell nuclei were counter stained with methyl green and cells photographed.

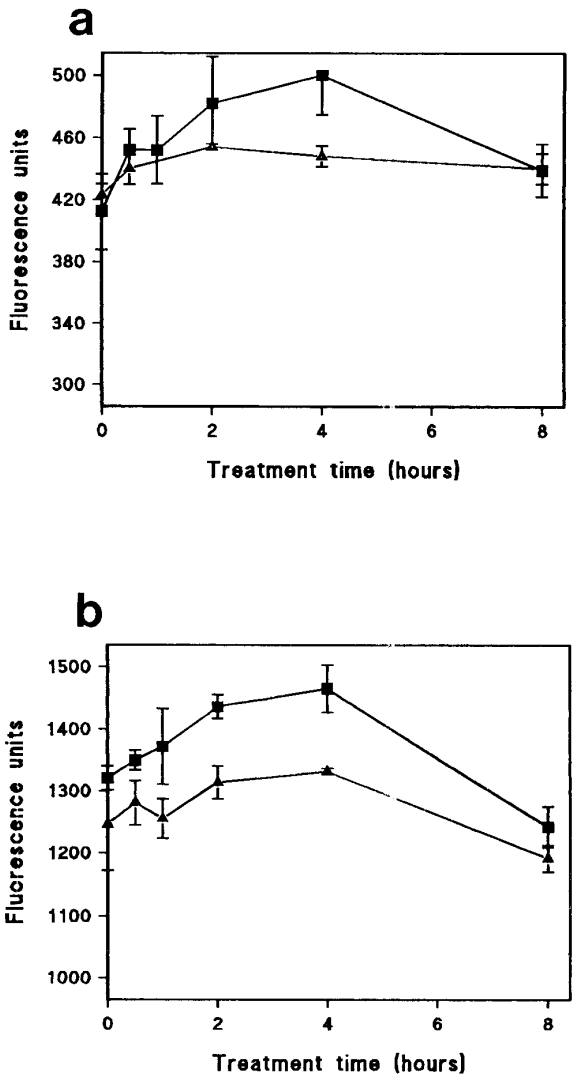

FIG. 1. Quantification of tumour cell attachment to HGF/SF-activated endothelium using DiI. Endothelial monolayers were incubated with HGF/SF (40ng/ml) for between 0.5 and 8 hours following which human tumour cells were allowed to adhere for 50 minutes. After washing, the numbers of cells remaining attached to the endothelium were quantified using a fluorescence plate reader. (a) HT115 cells, (b) MCF7 cells. ■, no CD44 antibody; ▲, CD44 antibody included (0.3mg/ml). Activation of endothelium with HGF/SF promotes tumour cell attachment, an effect that is blocked by inclusion of an anti-CD44 antibody.

RESULTS

Tumour cell attachment to endothelial cells. Following HGF/SF stimulation, the number of cancer cells attaching to the endothelium significantly increased, reaching maximal levels in samples in which the endothelium was incubated with HGF/SF for 4 hours (figure 1a, ■). Inclusion of an anti-CD44 antibody in this assay blocked the effect of HGF/SF and no significant rise in tumour cell adherence was seen following HGF/SF activation of the endothelium (figure 1a, ▲). Similar results were observed for the breast cancer cell line, MCF7 (figure 1b).

Adhesion molecule ELISA. Detection of cell-surface CD44 following HGF/SF stimulation was facilitated by an ELISA based method. Following culture of endothe-

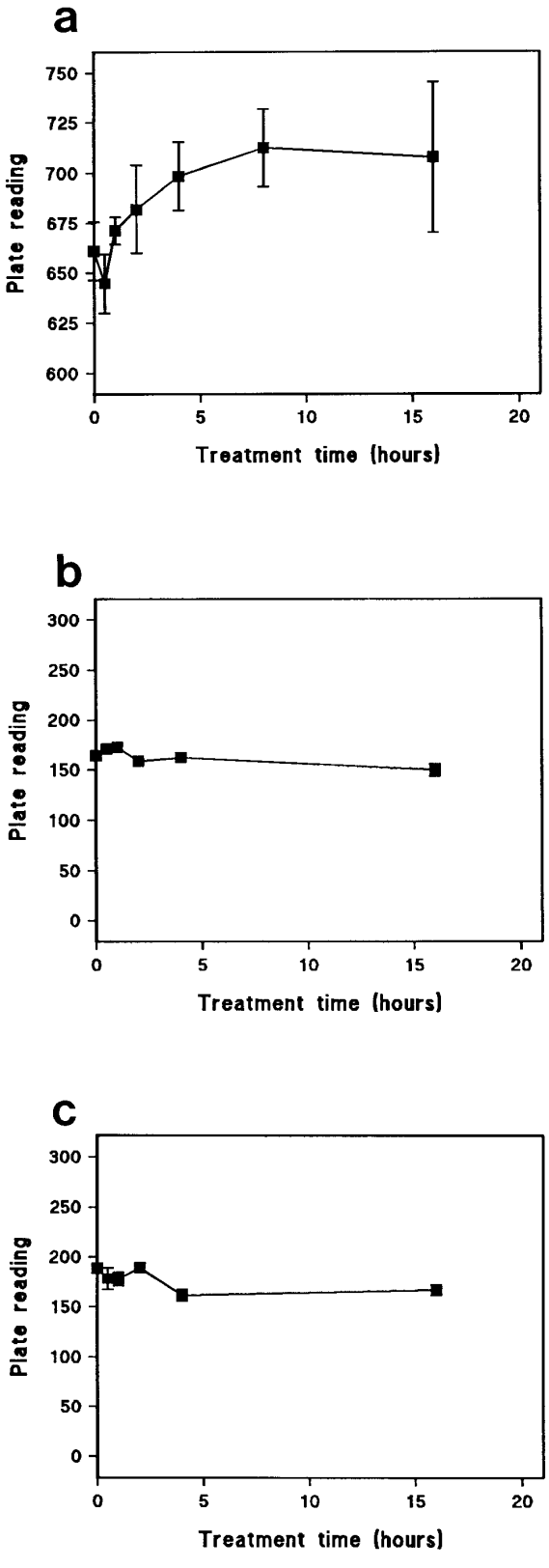

FIG. 2. Expression of endothelial cell surface adhesion molecules following HGF/SF stimulation. Following activation of endothelial monolayers in 96-well plates, expression of CD44, E-selectin, and VCAM-1 was determined using an ELISA. (a) CD44, (b) E-selectin, (c) VCAM-1. Levels of CD44 increase in response to HGF/SF stimulation, whereas E-selectin and VCAM-1 show no significant change.

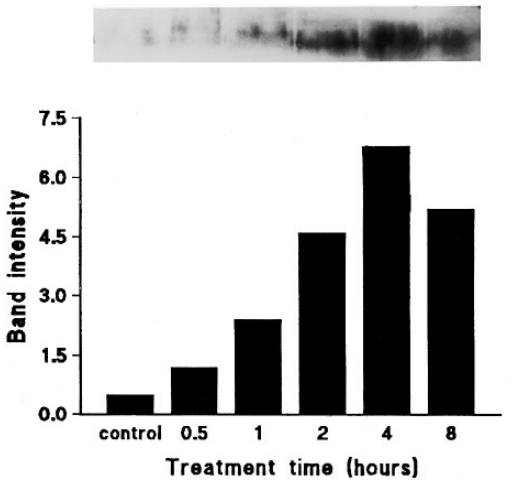

FIG. 3. Western blotting of cellular proteins. Following separation of total cellular proteins from HGF/SF-stimulated and unstimulated endothelial cells by 8% SDS-PAGE and subsequent electro blotting, membranes were probed for the presence of CD44. An 80kDa protein corresponding to mature CD44 was seen to increase in response to HGF/SF stimulation, reaching a maximum after 4 hours incubation.

lial cells with HGF/SF in 96-well plates and incubation with anti-CD44 antibodies, a colourimetric product was obtained by addition of OPD. An increase of CD44 was observed in cells treated with HGF/SF (figure 2a). Expression of VCAM-1 and E-selectin was also measured using similar techniques. Resting levels of these molecules were much lower than that of CD44, and were not seen to increase following cell treatment with HGF/SF (figure 2b-E-selectin, figure 2c-VCAM-1).

Western blotting. Following treatment of endothelial cells with hepatocyte growth factor for between 30 minutes and 8 hours, the amount of CD44 protein in these cells was determined by Western blotting as described above. Blots revealed the presence of an 80kDa protein corresponding to the mature CD44 product (figure 3). Expression of this protein was observed to increase in response to the length of HGF stimulation, reaching a maximum level following 4 hours stimulation. After 8 hours of culture with HGF levels of CD44 protein had fallen but were still higher than that of the control (not treated) sample.

Immunohistochemical staining of CD44. The expression of cell surface CD44 was determined in both untreated and HGF-stimulated endothelial cell monolayers by immunocytochemistry (cell nuclei were counter-stained with methyl green in both cases). En-

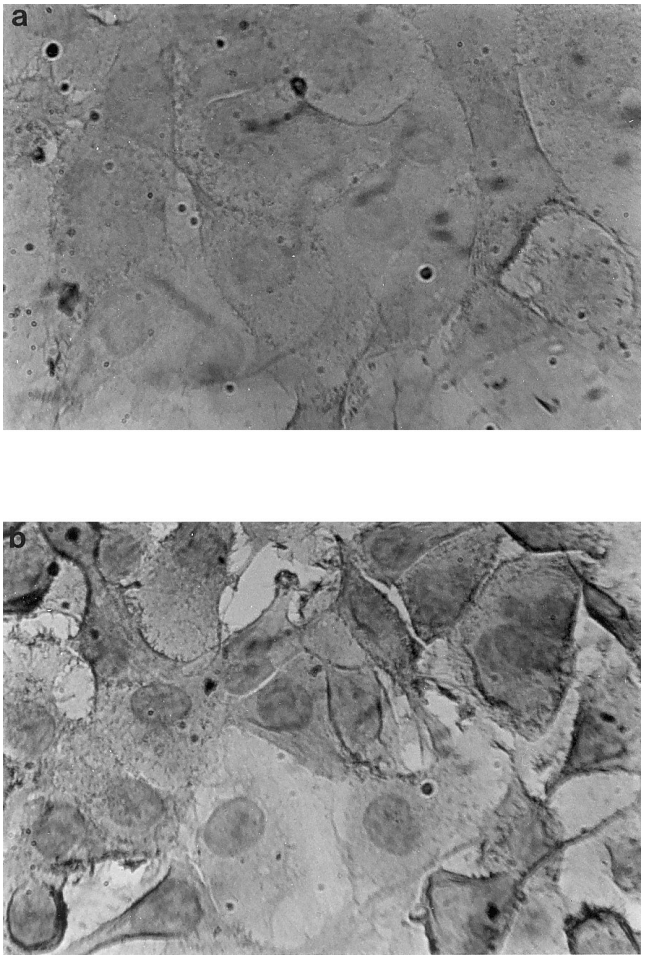

FIG. 4. Immunohistochemical staining of CD44 in normal and HGF/SF activated endothelium. Unstimulated endothelial cells (a) display a uniform, weak stain for CD44. Following 4 hours incubation with HGF/SF (b), the cells show a more intense staining, particularly at cell edges.

dothelial monolayers not treated with HGF/SF were observed as a compact monolayer, displaying a weak, uniform surface stain with visible areas of cell-cell contact (figure 4a). After 4 hours HGF/SF treatment these cells showed a more intense staining of CD44 on their surface, particularly at the cell edge (figure 4b).

DISCUSSION

The spread of tumours throughout the body can be considered to occur in a number of steps, collectively termed the metastatic cascade. A key stage of this process is the arrest of circulating tumour cells at capillary walls or within draining lymph nodes followed by extravasation and migration into the surrounding organ (5,6).

Metastasising tumour cells and activated lymphocytes posses comparable molecular properties and thus activities such as invasive behaviour, migration and extravasation occur via similar mechanisms. These cellular functions are dependant upon a number of cell-surface adhesion proteins and CD44 has been implicated as one such molecule (7,10).

CD44 (11) is a 80-120 kDa transmembrane protein originally implicated in the attachment of lymphocytes to high endothelial venules (HEV) and their subsequent migration into lymph nodes (12,13). CD44 is known to exist in many different isoforms arising by alternative splicing events (14). Recent studies have shown that the some isoforms may be involved in homotypic binding, aiding leukocyte extravasation (15) and the presence of yet others may correlate with enhanced metastatic function or tumour progression (16-18). It should be noted, however, that other studies suggest no correlation between CD44 presence and metastatic behaviour (19) so the exact role of CD44 in metastases formation is unclear.

It may be the case that CD44 plays a role in the initial capture of metastasising tumour cells passing over endothelium as it does to neutrophils during an inflammatory response or lymphocytes travelling through lymph nodes. This would then allow other adhesion molecules to come into play, establishing firmer contacts between these cells and the endothelium. Our study here shows that stimulation of endothelial monolayers with HGF/SF resulted in an up-regulation of CD44 expression together with enhancement of tumour cell-endothelial cell adherence. This increase in the number of tumour cells attaching to the activated endothelium could be attributed to increased CD44 expression as inclusion of anti-CD44 antibodies inhibited these effects. The effects of HGF/SF *in vivo* on endothelial cells has not been assessed, but preliminary results using HUVEC cells *in vitro* have shown similar results (data not shown).

Several studies have shown that CD44 promotes intracellular signal transduction events which may in turn regulate the expression of other adhesion molecules or growth factors by the cell (20,21). Although HGF/SF up-regulates the expression of CD44 on endothelial cells, no effect on other adhesion molecules involved in the stronger endothelium-tumour cell interactions, E-selectin and VCAM-1, could be detected.

It is interesting to note that a recent study by Kawakami *et al* (22) has shown that HGF/SF up-regulates the expression of tumour cell intergrin molecules, resulting in enhancement of their adhesive properties towards extracellular matrix components. These observations further implicate HGF/SF in the enhancement of tumour cell interactions necessary for successful metastasis.

We conclude that the increased avidity of metastatic cells for molecules expressed on the surface of the endo-

thelium may be promoted by the CD44 up-regulatory effects of HGF/SF and thus this factor may act to facilitate metastatic spread.

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REFERENCES

1. Matsumoto, K., and Nakamura, T. (1996) *J. Biochem.* **119,** 591–600.
2. Jiang., W. G., and Hiscox, S. (1997) *Histol. Histopathol.* **12,** in press.
3. Sunitha, I., Meighen, D. L., Hartman, D. P., Thompson, E. W., Byers, S. W., and Avigan, M. I. (1994) *Clin. Exp. Metab.* **12,** 143–154.
4. Jiang, W. G., Puntis, M. C. A., and Hallett, M. B. (1994) *Br. J. Surg.* **81,** 1576–1590.
5. Hart, I. R., Goode, N. T., and Wilson, R. (1989) *Biochim. Biophys. Acta* **989,** 65–84.
6. Van Roy, F., and Mareel, M. (1992) *Trends Cell Biol.* **2,** 163–167.
7. Herrlich, P., Zoller, M., Pals, S. T., and Ponta, H. (1993) *Immunol. Today* **14,** 395–399.
8. Hiscox, S., and Jiang, W. G., (1997) *Cancer Lett.*, in press.
9. Rice, J. W., Davis, J. E., Crowl, R. M., and Johnston, P. A. (1996) *Anal. Biochem.* **241,** 254–259.
10. Albelda, S. M. (1993) *Lab. Invest.* **68,** 4–17.
11. Tanabe, K. K., and Saya, H. (1994) *Crit. Rev. Oncol.* **5,** 201–212.
12. Underhill, C. (1992) *J. Cell Sci.* **103,** 293–298.
13. Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1989) *Cell* **56,** 1057–1062.
14. Yu, Q., and Toole, B. P. (1996) *J. Biol. Chem.* **271,** 20603–20607.
15. Droll, A., Dougherty, S. T., Chiur, K., Dirks, J. F., McBride, W. H., Cooper, D. L., and Dougherty, G. J. (1995) *J. Biol. Chem.* **270,** 11567–11573.
16. Yamaguchi, A., Saito, M., Gio, T., Iida, A., Takeuchi, K., Hirose, K., Nagawara, G., Urano, T., Furuwkawa, K., and Shiku, H. (1995) *Jpn. J. Cancer Res.* **86,** 1166–1171.
17. Mulder, J. W., Kruyt, P. M., Sewnath, M., Oosting, J., Seldenrijk, C. A., Weidema, W. F., Offerhaus, G. J., and Pals, S. T. (1994) *Lancet* **344,** 1470–1472.
18. Gansauge, F., Zobywalski, A., Scharnweber, C., Link, K. H., Nussler, A. K., and Beger, H. G. (1995) *Cancer Res.* **55,** 5499–5503.
19. Terris, B., Flejou, J. F., Dubois, S., Ruszniewshki, P., Scoazec, J. Y., Belghiti, J., Potet, F., Bernades, P., Mignon, M., and Henin, D. (1996) *J. Clin. Pathol. Mol. Pathol.* **49,** M203–M208.
20. Shimizu, Y., Van Seventer, G., Siraganian, R., Wahl, L., and Shaw, S. (1989) *J. Immunol.* **143,** 2457–2463.
21. Koopman, G., van Kooyk, Y., de Graaff, M., Meyer, C. J., Figdor, C. G., and Pals, S. T. (1990) *J. Immunol.* **145,** 3589–3593.
22. Kawakami-Kimura, N., Narita, T., Ohmori, K., Yoneda, T., Matsumoto, K., Nakamura, T., and Kannagi, R. (1997) *Br. J. Cancer* **75,** 47–53.