MARKER PROFILE OF MESOTHELIAL CELLS VERSUS OVARIAN CARCINOMA CELLS

C.C. van Niekerv3, P.H.K. Jap1, C.M.G. Thomas3, D.F.C.M. Smeets2, F.C.S. Ramaekers5 and L.G. Poels1,5

Departments of 1Cell Biology and Histology, 2Cytogenetics, 3Obstetrics and Gynaecology, and 4Pathology, Medical Faculty and St. Radboud Hospital, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

We investigated the marker profile of human ascitic and cultured mesothelial cells, and compared it to that of ovarian carcinoma cells which are related in terms of their histogenesis, unrelated colon carcinomas being used as reference. Mesothelial and ovarian carcinoma cells could not be distinguished by (intermediate) filament typing, using monoclonal antibodies (MABS) to keratins, vimentins and desmins. Colon carcinomas differed from mesothelial cells and ovarian carcinomas by the absence of keratin-7 filaments. The epithelial marker BW 495/36 was completely negative on mesothelial cells and positive on all ovarian and colon carcinoma cells. While CEA was found on about 85% of all colon carcinomas, CEA expression on mesothelial cells and ovarian carcinomas was below 20%. The ovarian carcinoma markers (OV-TL 3, OV-TL 10, OC 125, MOV 18) were strongly positive on ovarian carcinomas and negative on colon carcinomas (or limited to traces of immunofluorescence on some samples). Although the mesothelial cells showed weak or negative reactivity with these markers, OC 125 antigen was found by immunoelectron microscopy on the surface of cultured mesothelial cells, and was shed in the culture supernatant at concentrations of 50, 25 and 50/25 U/ml of 106 positive cells. This suggests that mesothelial cells may be responsible for the synthesis of CA 125 in ascitic fluid. The data indicate that ovarian carcinomas, mesothelial cells and colon carcinomas can be distinguished using a combination of anti-keratin antibodies with BW 495/36 and anti-ovarian carcinoma markers.

The human ovary is covered by a layer of cells called the "germinal epithelium". This layer consists of coelomic epithelial cells which also line the peritoneal surface. The ovarian surface epithelium (Blaustein, 1981) or ovarian mesothelioma (Woodruff, 1979) is the site of origin of the largest group of ovarian tumours. The formation of crypts, inclusion cysts, and papillary structures is considered to represent steps in differentiation, proliferation and possible malignant transformation of the coelomic ovarian epithelium, thought to be mediated through hormones (Cramer et al., 1983). Although ovarian mesothelioma as well as pelvic and extra-pelvic mesothelioma have a common embryonic origin, local factors and/or reproductive patterns may play a role in regulating growth and development of the mesothelium, and might explain structural morphological differences between ovarian mesothelioma and adjacent peritoneal mesothelioma (Nicosia and Johnson, 1984).

The distinction between malignant cells, atypical mesothelial cells, and mesothelial cells in ascitic fluid of patients with ovarian carcinoma can often present a difficult diagnostic problem (Jap et al., 1981). The marker profile of 2 related cell types (mesothelial cells and ovarian carcinoma cells) was compared to that of an unrelated tumour such as colon carcinoma, also frequently causing intraperitoneal ascites. We have thus investigated the reactivity of mesothelial cells with a panel of MABS to ovarian carcinoma-associated antigens, to intermediate filaments (keratin, vimentin, desmin), to the epithelial marker BW 495/36, and to CEA, and compared it to that of ovarian carcinoma and colon carcinoma cells.

MATERIAL AND METHODS

Patient data

Ascitic fluid was obtained from women with ovarian carcinoma. These ascitic samples contained ovarian carcinoma cells and mesothelial cells. Mesothelial cells were also collected from patients with ascites in which no malignant cells were diagnosed. Biopsies of ovarian carcinomas and colon carcinomas were snap-frozen and used for the immunofluorescence assay.

Culture method

The pelleted ascitic cells were resuspended in RPMI-1640-based culture medium (Gibco, Paisley, Scotland) and cultured as described for ovarian carcinoma cell lines (Poels et al., 1989). The mesothelial cells could be subcultured at a 1:2 split ratio, for a limited number of passages (7–10). Cell growth studies were carried out by culturing cells in plates containing 24-wells, 16 mm in diameter (Costar, Cambridge, MA) with a change of medium every 2 or 3 days. Cells were seeded at a density of 104 cells per ml per well. On subsequent days cells were detached with trypsin/EDTA and counted with a haemocytometer (van Niekerv et al., 1988). Triplicate cultures were used per 24 hr interval and the mean number of cells per ml per well was expressed on a logarithmic scale. Doubling time was calculated from logarithmic growth-phase cultures.

Light and electron microscopy

Cells grown on multipot slides (Flow, Irvine, Scotland) were fixed for 2 min in cold methanol (–20°C) and routinely stained with May–Grünwald/Giemsa and PAS reaction (in combination with standard diastase treatment) and with Alcian blue according to standard histological procedures.

For standard electron microscopy, monolayers of tumour cells, grown in 24-well plates (Flow), were fixed for 10 min in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 420 mM) at 4°C.

For immunoelectron microscopy the tumour cells were prefixed in periodate-lysine-paraformaldehyde-glutaraldehyde solution (PLPG), for 10 min (Gendelman et al., 1983), washed with PBS, then incubated with MABS and finally with rabbit anti-mouse IgG peroxidase (1:100, Dako, Copenhagen, Denmark). The coated tumour cells were post-fixed for 10 min in a 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4), stained with 0.1% diaminobenzidine-HC and 0.01% H2O2, and further processed for electron microscopy (Poels et al., 1989).

Indirect immunofluorescence

Reactivity of MABS was tested by the immunofluorescence assay described by Poels et al. (1986). Cells grown on multipot slides (Flow) were pre-fixed in cold methanol (–20°C) for 1–2 min, and tested by the immunofluorescence assay.

No difference was seen in the expression of the different markers on cold methanol pre-fixed and non-pre-fixed cells. The following sets of MABS were used:

To whom reprint requests should be sent.

Received: January 24, 1989.
A. Mouse MAbs against ovarian carcinoma: OC 125 (Bast et al., 1981); OV-TL 3 (Poels et al., 1986); OV-TL 10, cytoplasmic antigen of 60% of serous ovarian carcinomas; MOV 18 (Miotti et al., 1987).

B. Mouse MAbs against intermediate filaments: keratin-18: RGE 53 (Ramaekers et al., 1983); RCK 106 (Ramaekers et al., 1987); keratin-7: RCK 105 and OV-TL 12/30 (Ramaekers et al., 1987); broad-spectrum keratin: OV-TL 12/5 with a similar tissue-distribution pattern to that of RCK 102 (Ramaekers et al., 1987); vimentin: RV 202 (Ramaekers et al., 1987; Quax et al., 1985); and desmin: RD 301 (Quax et al., 1985).

C. Mouse MAbs BW 431/31 against a CEA epitope (Bosslet et al., 1985), and BW 495/36 against a 200-kDa glycoprotein in epithelial cell types (Bosslet et al., 1987). The ovarian carcinoma cell lines OTN 11 (Poels et al., 1989), OTN 14 (van Niekerk et al., 1988), EFO 21, EFO 27 (Simon et al., 1983), OAW 42 (Wilson, 1984), OVCA 433 (Bast et al., 1981), and OVCA 3 (Hamilton et al., 1983) were evaluated with the same set of MAbs as used for the mesothelial cell strain.

CEA and CA 125 levels in culture medium

Concentrations of CEA and CA 125 shed into the culture medium were determined in medium that had been changed every 2 days and was taken from cultures with a cell density varying between 10^4 and 10^6 cells/ml growing in 24-well plates. The CEA content of the supernatant was measured by an enzyme-immuno assay (ELA) (Abbott, North Chicago, IL) and the CA 125 level was measured by an immuno-radiometric assay (IRMA) (Centocor, Malvern, PA) according to the manufacturer's instructions.

Flow cytometry and cytogenetic analyses

Flow cytometric analyses of the DNA content of the mesothelial cells and cytogenetic analyses were carried out as described by van Niekerk et al. (1988).

RESULTS

Light and electron microscopy of cell cultures

The mesothelial cell strains adhered to plastic as well as to glass surfaces, growing initially as monolayers. They proliferated intensely and epitheloid sheets and confluent monolayers initially developed within 1–2 weeks. Tumour cells were rapidly overgrown by mesothelial cells and eventually degenerated. No contamination of the few ovarian carcinoma cells present in the ascitic fluid was observed after 1 week. In the later cultures only mesothelial cells appeared to survive as judged by phase-contrast microscopy and after Giemsa staining. An explicit distinction was to be made between these mesothelial cell cultures and ovarian carcinoma cell cultures. These mesothelial cells grew as large and single flattened cells as well as in islets (Fig. 1a). They did not form 3-dimensional clusters, even in regions of high cell density, while the smaller ovarian carcinoma cells grew rapidly, often overlapping in 3-dimensional clusters (van Niekerk et al., 1988). Mesothelial cell growth stopped after confluence was reached. Originally the ascitic, reactive mesothelial cells demonstrated strong diastase-sensitive PAS staining while the cultured cells appeared to lose their glycogen.

Electron microscopically, cultured mesothelial cells appeared to be connected by moderate amounts of small fascia-adherens-like junctions (Fig. 1b). Generally, in early subcultures, most cells showed a limited number of slender microvilli as well as irregular and blunt protrusions decreasing in later passages (Fig. 1b). Peripherally localized microfilaments and randomly-distributed intermediate-sized filaments were distinct (Fig. 1b,c). Well-developed organelles, lipid droplets and electron-lucent autophagic vacuoles of varying diameters were observed (Fig. 1c,d). Abundant glycogen particles were clearly demonstrated in the peripheral part of the cytoplasm during the first subcultures but in the later ones hardly any glycogen was detectable (Fig. 1c,d).

DNA flow cytometric and cytogenetic analysis of cultured mesothelial cells

Flow cytometric analysis of propidium-iodide-stained mesothelial cell strains (MC 1, 2, 3) indicated that the ploidy of the trypsinized mesothelial cells (of the 5th passage) was 1.1125, 1.114, and 1.168 respectively, approximating diploid status. In cytogenetic analyses the chromosome counts of 50 mitoses revealed a normal diploid (46) number of chromosomes in most of the cells in each culture (passage 5).

Immunocytochemical studies

Table I shows that all mesothelial cells in ascitic samples as well as in culture reacted strongly with all anti-keratin MAbs in the immunofluorescence assay. This confirms the epithelial character of the cells. The keratins showed a filamentous network in cultured mesothelial cell strains (Fig. 2a–e). The immunoperoxidase staining was confined to parts of the peripherally localized, thick bundles of filaments, as shown by immuno-electron microscopy (Fig. 1e,f). The reactivity of anti-keratin MAbs with ovarian carcinoma cells in tissue sections, in ascites, as well as in culture, was identical to the results described for mesothelial cells (Table I). Colon carcinomas were distinguished from mesothelial cells and ovarian carcinoma cells by the absence of reactivity with anti-keratin-7 antibodies (Table I).

The reactivity with the BW 495/36 MAb against a 200-kDa glycoprotein in epithelial cell types was negative, both in ascitic mesothelial cells (Table I) and in the 3 cultured mesothelial cell strains (Fig. 2g). This epithelial marker, however, was positively expressed on all ovarian carcinoma cells in sections, ascites and cell lines as well as on colon carcinomas (Table I). The muscle-specific intermediate filament desmin was found neither in mesothelial cells (Fig. 2h) nor in ovarian carcinoma cells (Table I). Vimentin was expressed in both ascitic and cultured mesothelial cells (Fig. 2f) as well as in ovarian carcinoma cells (Table I). Vimentin was not found in sections of ovarian carcinomas.

The ovarian carcinoma markers, defined by the OV-TL 3 and MOV 18 MAbs, were weakly expressed on mesothelial cell strains (Figs. 2h and 2i), and the expression was similar on ascitic mesothelial cells (Table II). These antibodies reacted strongly with ovarian carcinoma cells in tissue sections and

FIGURE 1 – One light and 6 electron micrographs of mesothelial cell strains. (a) Third passage of pure mesothelial cells (MC): monolayer at beginning of confluence (phase-contrast). Scale bar: 50 μm. (b) Detail of 2 mesothelial cells with fascia adherens-like junctions (asterisk), thick bundle of peripherally located filaments (thick arrow) and a few long, slender microvilli (5th passage) (thin arrow). Scale bar: 0.4 μm. (c) First passage: a mesothelial cell with well-developed organelles, autophagic vacuoles (asterisk), lipid droplets and intermediate-sized filaments. Note area with glycogen granules (arrows) preferentially located in the outer zone as well as within the blunt protrusions. Scale bar: 0.4 μm. (d) Detail of a mesothelial cell in the 5th passage with numerous organelles, fat droplets (asterisks), and dispersed intermediate-sized filaments (arrows). Scale bar: 0.4 μm. (e) Positive immunoperoxidase staining (arrows) of peripherally located filaments with the OV-TL 12/30 MAb (cytokeratin-7). Scale bar: 0.4 μm. (f) High magnification of positive immunoperoxidase-stained filaments with the OV-TL 12/5 MAb (cytokeratin-5 + 7 + 14 + 19). Scale bar: 0.4 μm. (g) Detail of positive immunoperoxidase-stained surface and microvilli of a mesothelial cell (arrows) with the OC 125 MAb (against ovarian carcinoma). Cytoplasmic structures react negatively. Scale bar: 0.4 μm.
TABLE I – EPITHELIAL MARKER PROFILE OF MESOTHELIAL, OVARIAN CARCINOMA AND COLON CARCINOMA CELLS

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<th>RGE 125</th>
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<th>BW 495/36</th>
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<td>Ascites mesothelial cells (n = 6)</td>
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<td>Mesothelial cell strains</td>
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<td>Ovarian carcinoma cell lines</td>
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<td>Ovarian carcinoma tissue sections (n = 60)</td>
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<td>Colon carcinoma tissue sections (n = 10)</td>
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1 Only 5-10% of the cells were strongly positive, the majority being negative. The fluorescence intensity as well as the number of positive cells were arbitrarily scored as: -- negative, ± weakly positive, + positive, ++ strongly positive. ND: Not done. -- Samples obtained from 6 patients with ascites in which ovarian carcinoma cells were observed. -- In one sample some sporadic cells were positive.

 ascites; the reactivity on ovarian carcinoma cell lines, however, was variable (Table II). The OC 125 and OV-TL 10 MAbS were found on less than 10% of the ascitic and cultured mesothelial cells staining with varying intensities (Fig. 2/k). The OC 125 antibody label was located at the cell surface of the mesothelial cells as shown by immunoelectron microscopy (Fig. 1g). OC 125 reacted positively with ovarian carcinoma cells in tissue sections and ascites (Table II). OV-TL 10 was positive on 30 out of the 50 ovarian carcinoma samples. The reactivity of all these MABs with ovarian carcinoma cell lines was variable, as shown in Table II, and negative on colon carcinomas.

The anti-CEA BW 431/31 MAb reacted heterogeneously on all examined samples, and did not discriminate between mesothelial cells, ovarian carcinoma cells and colon carcinoma cells (Table II).

No difference was seen in the expression of the different markers when cells were fixed briefly (1-2 min) in cold methanol (−20°C).

CMA and CA 125 levels in the culture medium of mesothelial cells

At passage 3, the supernatants of a growth-culture experiment with a long doubling time (5-6 days) during logarithmic growth were tested for CA 125 and CMA on day 1 and day 17 during growth. In all day-1 samples of the 3 cell strains, CA 125 activity remained low at the level of 15 Ulml, being similar to that seen in the control culture medium. At day 17, CA 125 activity was 25 Ulml and 28 Ulml (strain MC 2, 3) and in the third mesothelial cell strain (MC 1) the CA 125 level increased to 50 Ulml in 2 days, while the cell density increased from 1-2 × 10^6 to 1-2 × 10^7 cells. The amount of CMA produced in the same culture samples remained below the control level of 1.2 µg/l of the control culture medium.

DISCUSSION

In a search for criteria to differentiate mesothelial cells and related ovarian carcinomas, we have investigated some cell characteristics of ascitic mesothelial cells as well as 3 mesothelial cell strains isolated from the ascites of patients with ovarian carcinoma. The marker profile of these mesothelial cells was compared to that of ovarian carcinomas and of colon carcinomas. In culture, mesothelial cells appear to be epitheloid with a limited life-span, but they could be subcultured at least 7-10 times. When confluence was reached, cell growth stopped. Limited growth potential and normal DNA index (FCM) analyses as well as a normal karyotype pattern, are not sufficient to allow a distinction to be made between mesothelial and ovarian carcinoma cells. Immunocytochemical characterization using MABs to different keratins (5/8/18/7) did not reveal any distinct immunoreactivity pattern for mesothelial versus ovarian carcinoma. This confirms and extends the data of Wu et al. (1982), using a different panel of anti-keratin MABs. The strong expression of vimentin on cultured mesothelial cells (Connell et al., 1983; LaRocca and Rheinwald, 1984), and the co-expression of vimentin with keratin can be considered as being characteristic of mesothelial cells in vivo (van de Molengraft et al., 1986). Initiation of vimentin expression is thought to be an adaptation to cell culture conditions, both in vitro and in vivo, rather than being indicative of mesenchymal cell origin (Franke et al., 1979). The absence of muscle-specific desmin filaments was used as a negative control for ascertaining the epithelial character of isolated mesothelial or ovarian carcinoma cells. Although typing with antibodies to intermediate filaments did not permit a distinction to be made between mesothelial and ovarian carcinoma cells, the BW 495/36 MAb appeared to discriminate between mesothelial cells reacting 100% negatively and ovarian carcinoma cells reacting 100% positively in ascites as well as in vitro. BW 495/36 is directed against a 200-kDa glycoprotein (Bosslet et al., 1987) not present on mesothelioma cells (Bosslet, personal communication). This means that within a keratin-positive as
|                        | OV-TL 3 | OV-TL 10 | OCl25 | MOV18 | CEA
|------------------------|---------|----------|-------|-------|-----
| Ascitic mesothelial cells | +       | +        | -/+   | -/+   | -/+ <10%<sup>1</sup>
| (n = 6)                |         |          |       |       |     
| Mesothelial cell strains MC 1, 2, 3 | +       | +        | +/+   | +     | +     
| Ascitic ovarian carcinoma cells | +/+     | +/+      | +/+   | +/+   | +     
| (n = 15)               |         |          |       |       |     
| Ovarian carcinoma<sup>3</sup> cell lines |         |          |       |       |     
| OTN 14                 | +       | +        | +     | +     | +     
| OTN 11                 | +       | +        | +     | +     | +     
| EPO-21                 | -/+     | +        | +     | +     | +     
| OAW-42                 | -/+     | -/+      | +     | +     | +     
| OVCA 433               | +/+     | +        | +     | +     | +     
| OVCAR 3                | +/+     | +        | +     | +     | +     
| Ovarian carcinoma tissue sections | +/+     | +        | +     | +     | +     
| (n = 10)               |         |          |       |       |     
| Colon carcinoma tissue sections | -<sup>=</sup> | -<sup>=</sup> | 65/65 | 30/50 | 42/50 | 1/10 | 9/10 |

The fluorescence intensity as well as the number of positive cells were arbitrarily scored as: - negative, + weakly positive, +/+ positive, +/+ strongly positive. ND: not done or less than 10% of the cells reacted 2+ or 3+, the majority being negative. Samples obtained from 6 patients with ascites in whom no malignant cells were diagnosed. Samples obtained from 15 patients with ascites in whom ovarian carcinoma cells were observed. Traces were found in 3 samples. Traces were found in all samples. Traces were found in 1 sample.

The results indicate that mesothelial cells can be excluded on the basis of their absence of reactivity with the BW 495/36 MAb.

Concerning the colon carcinomas, BW 495/36 reacted positively with all samples. In contrast to mesothelial and ovarian carcinoma cells, colon carcinomas and normal colon tissue sections (results not shown) did not express keratin 7. It is thus possible to distinguish reliably between mesothelial, ovarian carcinoma and colon carcinoma cells. With regard to the evaluation of ovarian carcinoma markers, mesothelial cells in ascites, as well as in culture, reacted weakly or not at all with all MABs to ovarian carcinoma cells (OV-TL 3, OV-TL 10, OC 125, MOV 18). All ovarian carcinoma cells in sections or ascites were strongly positive with one or more of these MABs. However, several ovarian carcinoma cell lines largely lost their reactivity with these markers after growth in culture, thus restricting the value of ovarian carcinoma markers mainly to in vivo conditions. Since colon carcinomas also reacted negatively or very weakly, strong positive staining with ovarian carcinoma markers on tumour cells is considered as a primary indicator of ovarian carcinomas.

We have substantiated the suggestions of Kabawat et al. (1983), Bast and Knapp (1984) and Bergmann et al. (1987) that CA 125 might be synthesized by peritoneal cells, by showing that a low but significant amount of CA 125 antigen was shed in the culture supernatants of mesothelial cells. In addition, we showed by immunoelectron microscopy that CA 125 antigen was located on the surface of several mesothelial cells. For differential cytodiagnosis in ascites, CA 125 positive mesothelial cells can be distinguished by their negative reactivity with the BW 495/36 marker.

Reports on the presence of CEA on mesothelial cells, mesotheliomas and ovarian carcinomas are conflicting. Few mesotheliomas exhibiting CEA-positive staining have been described (Silcock et al., 1986; Walts et al., 1983). Absence of CEA epitopes on mesotheliomas as well as reactive mesothelial cells have also been reported by Marshall et al. (1984), Faravelli et al. (1985) and Heyderman et al. (1985). Although BW 431/31 MAb does not react with CEA-cross-reactive antigens, we found that it strongly stained a minority (20%) of cultured mesothelial cells. The in vitro incidence of CEA-positive mesothelial cells might reflect differentiating and proliferative state of cells by analogy with the expression of CEA in transitional epithelial cells (Shevchuck et al., 1981). The CEA marker could not distinguish between mesothelial, ovarian carcinoma and colon carcinoma cells. In contrast to the ovarian carcinoma cell line OTN 14, mesothelial cells in culture did not shed CEA antigen in the supernatant. This directly supports the suggestion of Faravelli et al. (1985) that CEA, although present in adenocarcinomas, is not always released in measurable amounts in effusions.

**ACKNOWLEDGEMENTS**

We are indebted to Dr. P. Kenemans and co-workers at Nijmegen, Amsterdam, for their cooperation in providing biopsy material and to Mr. M.F.G. Segers for his support in the CEA and CA 125 assays. We thank Mr. G. Merkx for cytogenetic analyses, Ms. A. Willemen for electron microscopy, Ms. K. Makkink and Dr. O.C. Boerman for help in the marker studies, and Mr. J. Koedam for assistance in animal experiments. We are grateful to Dr. K. Bosset (Behringwerke, Marburg, FRG) for providing the antibodies BW 431/31 and BW 495/36, to Dr. V.R. Zurawski, Jr. (Harvard Medical School, Boston, MA) for providing the OC 125 antibody and to Dr. M.I. Colnaghi (Milan, Italy) for providing the MOV 18 antibody.

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