Ovarian cancer is diagnosed at advanced stages in approximately 75% of patients. The prognosis in ovarian cancer remains poor, and there is a need to identify patients less likely to respond to treatment or patients who suffer from low-stage disease and have a worse prognosis. Early detection of ovarian cancer or of recurrent disease after therapy is very important in ovarian cancer patients. Serum tumour markers, which have been shown to be useful in early detection of primary tumours, recurrences, evaluation of prognosis and monitoring of therapy, have become an integral part of follow-up schemes in the management of ovarian cancer patients (Tarin and Matsumura, 1993; Dall et al., 1994; Gold and Osbahd, 1994).

The surface glycoprotein CD44 is widely distributed in different tissues (Flanagan et al., 1989). CD44 cell-surface adhesion molecules have been shown to mediate cell–cell and cell–matrix interactions (Screaton et al., 1992), allowing circulating lymphocytes to migrate into lymphatic tissue (Jalkanen et al., 1986; Salles et al., 1993) and to control lymphocyte binding to peripheral lymph nodes, mucosal and synovial endothelial cells (Jalkanen et al., 1987a). The CD44 family of transmembrane receptor molecules is derived from a single gene located on chromosome 11 (Koopman et al., 1993). By the mechanism of messenger RNA alternative splicing numerous isoforms of the CD44 glycoprotein are produced (Smith et al., 1989; Matsumura and Tarin, 1992; Screaton et al., 1992). Cell-surface proteins encoded by splice variants of CD44 differ from the CD44 standard isoform by additional amino acids in the extracellular region of the protein (Screaton et al., 1992). CD44 isoforms encoded by splice variants occur on the surface of different normal cells (Mackay et al., 1994). Tumour samples show a more complex pattern of CD44 expression, indicating a loss of splice control in malignantly transformed cells (Heider et al., 1993).

Rudy et al. (1993) could show that the expression of variants of the CD44 surface glycoprotein confers metastatic behaviour to a non-metastatic cell line in a rat carcinoma model (Gunthert et al., 1991). In colorectal cancer, breast and cervical cancer as well as in gastrointestinal lymphoma and gastric cancer a correlation between the overexpression of specific isoforms of CD44 and poor prognosis was reported (Joensuu et al., 1993a, b; Mayer et al., 1993; Wielenga et al., 1993; Kainz et al., 1995a).

It was speculated that the overexpression of specific CD44 isoforms, mainly CD44 splice variants v5, v6 and v7–8, could raise the metastatic potential of a tumour by facilitating the expansion of malignant cells into the draining lymph nodes (Salles et al., 1993).

Antibodies raised against different soluble CD44 molecules allow the detection of CD44 isoforms in serum samples. The measurement of soluble CD44 molecules in body fluids could be useful in early diagnosis of cancer, assessment of disease status and evaluation of metastatic potential and prognosis. In a recent study we investigated the serum levels of proteins encoded by CD44 splice variants in cervical cancer patients and found significantly elevated levels of CD44v6 when tumour was present (Kainz et al., 1995b). AG Zeimet et al. (personal communication) describe high serum levels of the CD44 standard molecule in ovarian cancer patients when tumour is present.

This prompted us to evaluate the levels of CD44 glycoproteins encoded by CD44 standard and CD44 splice variants v5 and v6 in serum samples of women suffering from ovarian cancer. Furthermore, we evaluated the immunohistochemical expression of epitopes encoded by CD44 splice variants v5, v6 and v7–8 in tumour specimens from the same sample of patients.

Patients and methods

This study includes 134 clinical and serological examinations of 22 patients suffering from ovarian cancer FIGO stages I–IV. All patients were treated by hysterectomy, lymph node sampling and omentectomy followed by a platinum-containing chemotherapy. All patients underwent a close follow-up programme consisting of regular visits at 3 month intervals, including vaginorectal palpation and ultrasonic examination of the lower abdominal tract. Computer tomography of the pelvis was performed every 6 months. Serum samples were taken before therapy and during a follow-up period of at least 12 months and up to 36 months and were stored at −20°C.
The patients were selected randomly. One preoperative serum sample and a minimum of four post-operative serum samples were available from every patient. In cases of tumour relapse at least five post-operative serum samples before the time of clinical evidence of relapse were available.

**Serum assay**

Serum levels of soluble CD44 isoforms CD44standard, CD44v5 and CD44v6 were measured using the sCD44standard (st) enzyme-linked immunosorbent assay (ELISA), sCD44v5 (v5) ELISA and sCD44var (v6) ELISA (Bender MedSystems, Vienna, Austria). All tests were run in duplicate according to manufacturer's instructions and without knowledge of the clinical outcome. A panel of 22 sera from healthy blood donors were tested for serum levels of CD44standard, CD44v5 and CD44v6.

To define the specificity of these ELISAs several structurally related and non-related polypeptides were tested for cross-reactivity. There is no detectable cross-reactivity with CD44 polypeptides lacking the protein sequence encoded by the corresponding exons. The limit of detection of soluble CD44 molecules (sensitivity) by antibodies used in these experiments was determined to be between 0.07 and 0.22 ng ml⁻¹. Intra-assay and inter-assay reproducibility has been calculated to be between 3% and 5.8%.

**Immunohistochemistry**

A total of 22 routinely formalin-fixed and paraffin-embedded surgery samples were used to evaluate the expression of epitopes encoded by CD44 splice variants CD44v5, CD44v6 and CD44v7-8. The paraffin sections were soaked in xylene to remove paraffin and rehydrated in a graded alcohol series (100–70%) and then rehydrated twice in xylene (Bio Genex, San Ramon, CA, USA) twice for 20 min in a microwave at 600 W power (HM 146, Elektra Bregenz, Schwaz, Austria) and then the sections were washed in 10 mM phosphate-buffered saline (PBS) (pH 7.6). Three different primary antibodies to different epitopes encoded by CD44 splice variants were used. The first was a monoclonal antibody specific for the epitope encoded by exon v6 of human variant CD44 (CD44v5, clone VFF-8, Bender), the second monoclonal antibody was specific for the epitope encoded by exon v6 of human variant CD44 (CD44v6, clone VFF-7, Bender) and the third monoclonal antibody was specific for the epitope encoded by exons v7–8 of human variant CD44 (CD44v7–8, clone VFF-17, Bender). The primary antibody was diluted in serum/PBS and the sections were incubated for 60 min and then incubated for 30 min with biotinylated anti-mouse and anti-rabbit link antibody (Dako LSAB 2 Kit, Dako, Carpinteria, CA, USA). After rinsing in PBS the sections were coated with streptavidin conjugated to alkaline phosphatase for 10 min. The sections were then rinsed in PBS, incubated with fast red chromogen (naphthol phosphate substrate in Tris buffer, fast red chromogen tablets 5 mg, Bio Genex, San Ramon, CA, USA) and then washed with distilled water. The sections were finally counterstained with haematoxylin and mounted. We interpreted strong and/or widespread staining as positive, weak and focal staining as negative.

**Positive control**

The positive control slide was prepared from epidermal tissue known to contain the antigen. In the positive control tissue all monoclonal antibodies stained similarly.

**Negative control**

The negative control slide was prepared from the same tissue block as the specimen. Instead of the primary antibody we used a normal, non-immune serum supernatant from the same source as the primary antibody.

**Statistics**

All results are expressed as mean ± standard error of the mean. Depending on the type and distribution of the data groups, comparisons were made by analysis of variance (ANOVA) procedures or by Wilcoxon two-sample test. The significance level assumed was P = 0.05.

**Results**

Mean age of the patients at the time of diagnosis was 57.6 ± 16.7 (range 26–89) years. Ovarian cancer stages I, II, III and IV were present in three, five, ten and four cases. Serous, mucinous cystadenocarcinoma, undifferentiated carcinoma and adenocarcinoma were present in nine, eight, three and two cases respectively. Our material included 76 and 58 serum samples of women with and without evidence of disease respectively.

The detected mean levels of healthy donors were 443 ± 125 ng ml⁻¹, 35 ± 13 ng ml⁻¹ and 170 ± 54 ng ml⁻¹ for CD44standard, CD44v5 and CD44v6 respectively. For CD44standard mean serum levels in patients with clinically detectable or clinically non-detectable ovarian cancer were 422.4 ± 143.8 ng ml⁻¹ and 547.4 ± 148.2 ng ml⁻¹ respectively (P-value not significant). For CD44v5 we measured a mean serum concentration in serum samples with clinically detectable tumour or not of 12.3 ± 7.9 ng ml⁻¹ and 21.9 ± 12.2 ng ml⁻¹ respectively (P-value not significant). Proteins encoded by the splice variant CD44v6 showed a mean concentration of 105.5 ± 37.9 ng ml⁻¹ when tumour was present and a mean concentration of 144.9 ± 50.9 ng ml⁻¹ in cases of complete remission (P-value not significant).

Serum levels of CD44 isoforms CD44standard, CD44v5 CD44v6 and CD44v7, grouped by the following clinical features: before therapy, complete remission, partial remission, steady disease and progression, are shown in Table 1. We found no significant differences between mean serum levels when pre-treatment samples were grouped by stage, histological type of tumour and lymph node involvement. Eight patients developed recurrent disease after complete remission. No increase of serum levels of CD44st, CD44v5 and CD44v6 could be observed before clinical evidence of relapse.

**Immunohistochemistry**

CD44 surface proteins containing epitopes encoded by splice variants CD44v5, CD44v6 and CD44v7–8 were detected by means of immunohistochemistry in two, three and one case of the 22 tumour samples respectively. We found homogeneous staining in tumours that were considered positive for CD44 expression. We found no correlation between expression of any of the splice variants and histological type, stage of the tumour or age of the patient.

**Table 1** Mean serum levels of CD44standard, CD44v5, CD44v6 and CD44v7 grouped by clinical status expressed as mean ± s.d.

|             | Before therapy | Complete remission | Partial remission | Steady disease | Progression |
|-------------|----------------|--------------------|-------------------|---------------|-------------|
| CD44standard (ng ml⁻¹) | 468 ± 2 ± 137.6 | 547 ± 2 ± 148.2 | 359.1 ± 106.2 | 371.3 ± 132.9 | 548.8 ± 142.1 |
| CD44v5 (ng ml⁻¹) | 14.6 ± 12.2 | 22.0 ± 12.2 | 12.3 ± 4.0 | 12.6 ± 5.8 | 9.5 ± 9.5 |
| CD44v6 (ng ml⁻¹) | 111.1 ± 46.8 | 144.9 ± 50.9 | 110.1 ± 32.0 | 99.9 ± 27.3 | 93.7 ± 46.5 |
| CD44v7 (ng ml⁻¹) | 2.5 ± 3.2 | 1.6 ± 3.1 | 2.3 ± 3.7 | 0.2 ± 0.6 | 0.3 ± 0.7 |

Mean serum levels of CD44standard, CD44v5, CD44v6 and CD44v7 grouped by clinical status expressed as mean ± s.d.
Discussion

In a previous study we were able to show that in cervical cancer patients' elevated CD44v6 serum levels were significantly correlated with clinical evidence of disease (Kainz et al., 1995b). CD44 isoforms CD44v5 and CD44v7-8 were strongly expressed by the tumour cells in cervical cancer patients. The expression of the CD44 isoform CD44v6 on tumour cells has been reported to be an independent prognostic factor in surgically treated cervical cancer patients (Kainz et al., 1995c). Although the two aforementioned studies examined different patient collectives, the available data lead to the conclusion that the expression of CD44v6 on tumour cells is reflected in its serum level.

In the present study we show that in this ovarian cancer collective the immunohistochemically detected expression rate of the CD44 isoforms CD44v5 and CD44v6 is very low. Consequently, serum levels of CD44v5 and CD44v6 in preoperative samples are not elevated compared with healthy controls.

Aberrant expression of glycoproteins encoded by CD44 splice variants has been detected by means of immunohistochemistry in human paraffin-embedded tumour samples (Heider et al., 1993; Tanabe et al., 1993). The expression of specific CD44 isoforms has been shown to be associated with metastasis and to be of prognostic relevance in human malignancies such as colorectal cancer, breast and cervical cancer, gastrointestinal lymphoma and gastric cancer (Joensuu et al., 1993a; Kainz et al., 1995a; Mayer et al., 1993; Wielenga et al., 1993).

Our data show that in ovarian cancer serum levels of the CD44 isoforms CD44v5 and CD44v6 do not reflect the tumour burden. Confirming that finding, we were able to show, by means of immunohistochemistry, that epitopes encoded by CD44 splice variants CD44v5, CD44v6 and CD44v7-8 are detectable in very low amounts in paraffin-embedded tissue samples of ovarian cancer patients. No correlation with any clinico-histological parameter was found.

When grouped by clinical status the highest serum levels of the CD44 standard isoform were found in cases of tumour progression and in cases of complete remission. This is surprising since pretherapeutic serum levels of CD44standard are not significantly elevated and thus serum levels of CD44standard does not seem to correlate with the presence of tumour.

All patients in our collective underwent a post-operative chemotherapy with six cycles of carboplatin (400 mg m⁻²) and cyclophosphamide (600 mg m⁻²) in a 4 week interval. In the cases of tumour relapse or progression second-line chemotherapy with paclitaxel (17 mg m⁻²) was applied after a 3 week interval. In order to stimulate peripheral blood progenitor cells patients were given granulocyte-macrophage colony-stimulating factor (GM-CSF; 5 μg kg⁻¹) (de Vries et al., 1991).

The CD44 standard isoform is distributed on haematopoietic cells, including all subsets of leucocytes, monocytes and erythrocytes and on different non-haematopoietic cells (Jalkanen et al., 1987a; Flanagan et al., 1989; Underhill, 1992). High-dose chemotherapy with the support of peripheral blood progenitor cells (PBPCs) is increasingly used in the treatment of solid tumours. Inducing PBPC proliferation by colony-stimulating factors results in up-regulation of CD34 expression. These cells do also express the CD44 standard isoform in almost 95% (Mohle et al., 1993; Dicke et al., 1994).

An increase of haematopoietic cells expressing the CD44 standard isoform could be responsible for the elevated CD44st serum levels.

We conclude that CD44 isoforms CD44standard, CD44v6 and CD44v7-8 are expressed at a very low level in malignant ovarian tumours. Serum levels of soluble CD44 isoforms do not reflect the tumour burden. Serum CD44standard showed higher levels when high haematopoietic activity was present. Therefore we hypothesise that the serum CD44standard production in haematopoietic cells overrules the production of ovarian tumour cells. The serum level of CD44standard mostly reflects the haematopoietic activity in these patients.

To clarify this matter we are currently examining the influence of growth factors used in chemotherapy regimens on the expression of CD44 isoforms.

Acknowledgements

This work was supported by a grant from the mayor of Vienna to Gerhard Sliutz.

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