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Modulation of Tumour Marker CA-125 Expression in Cultured Ovarian Carcinoma Cells

C. Marth, A.G.Zeimet, G. Böck and G. Daxenbichler

The aim of this study was to elucidate whether proliferation of ovarian carcinoma cells may affect the biosynthesis and release of CA-125. In a cell culture model the tumour marker CA-125 expression in cytosol, surface membrane, and release into culture medium was studied in six human carcinoma cell lines. Cell cycle analysis of propidium iodide stained nuclei was performed using a fluorescent activated cell sorter. The turnover of CA-125 is very rapid, within 24 h the equivalent amount found in each cell was also released in the supernatant culture medium. A good relation between cytosolic, membrane, and released CA-125 was observed. CA-125 expression was associated predominantly with the G_0/G_1 phase of the cell cycle and was dependent on cell density. The results presented here demonstrate that factors associated with tumour cell proliferation could influence the CA-125 serum level in ovarian cancer patients.
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INTRODUCTION

THE CA-125 TUMOUR marker, an antigenic determinant which is recognised by the monoclonal antibody OC125 is associated with high molecular weight glycoproteins [1]. These antigens are expressed by more than 80% of non-mucinous epithelial ovarian tumours as well as by other tissues of Müllerian origin [2, 3]. Due to its clinical value in pre-operative diagnosis and monitoring of ovarian cancer, CA-125 is the leading tumour marker in this disease. The function of the glycoproteins carrying CA-125 is unknown and, because of their complex nature, only little information about the physical and immunological properties of these antigens is available. It is important to know the factors regulating the expression of CA-125 to be able to further evaluate the definite role of CA-125 in monitoring patients. It is well known that CA-125 serum levels are closely related to tumour mass. The level of the tumour marker is elevated in only a small proportion of patients with microscopic disease, but in more than 70% of patients presenting with a tumour mass greater than 1 cm [4, 5]. A persistently rising serum CA-125 concentration is commonly associated with progression of the disease and is frequently observed several months prior to clinical evidence of progression. Apart from the number of tumour cells, other factors possibly influencing the level of CA-125 are still unknown. The aim of our study was to elucidate whether proliferation of tumour cells may affect the biosynthesis and release of CA-125. Moreover, we were interested in the kinetics of CA-125 turnover and its interactions with expression-inducing agents, such as interferons [6].

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MATERIALS AND METHODS

Cells

The human ovarian carcinoma cell lines OVCAR-3, HTB-77, SKOV-6, SKOV-8, 2774, and the human endometrial carcinoma cell line SKUT-2 were kindly provided by Dr C. Dittrich (Vienna) and Dr. G. Gastl (Memorial Sloan Kettering Cancer Center, New York) and were cultured as monolayer under the conditions described previously [6]. Briefly, the cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). To observe proliferation, cells from stock flasks were detached by trypsin-EDTA and about 50 000 cells were seeded in Nunc 24-well tissue culture plates. This was the time point 0 (T_0). The cells were allowed to attach for 24 h and then the culture medium was renewed. After a culture period of 7 days (T_1), cells were enumerated and mean population doubling time (PDT) was calculated using the following formula:

$(T_1 - T_0)/[\log(\text{no. of cells } T_1) - \log(\text{no. of cells } T_0)]/\log 2.$
$T_1 - T_0$ was expressed in hours.

CA-125 surface expression

This was assessed by a living cell radioimmunoassay as described by Greiner *et al.* [7]. First, the cells were seeded in 96-well tissue culture plates and after a varying period of time (cf. respective figures) the culture medium was aspirated and 100 µl of MEM containing 10% of bovine serum albumin (BSA) were added and incubated at 37°C for 1 h. Thereafter, the plates were washed once with MEM containing 1% BSA. The antiserum OC125 was diluted 1:3 with MEM containing 1% BSA, added to each well and incubated for 1 h. Subsequently, the wells were washed twice using MEM containing 1% BSA, and then 75 000 cpm of ^{125}I-labelled anti-mouse immunoglobulin F(ab')2 fragment were added. After incubation for 1 h, the wells were washed three times, and the cells were lysed by adding 100 µl of 2N sodium hydroxide to each well. The resulting solution was absorbed with a cotton tip and then analysed in a gamma scintillation counter (Berthold, Wildbad, Germany). Background counts (approximately 200–400 cpm) determined by means of a monoclonal antibody (Negative Control from Immunotech S.A., Marseille, France) were subtracted from those measured by using the OC-125.

Detection of shed CA-125

The ovarian carcinoma cells were grown in 24-well tissue culture plates containing the medium described above. The cell monolayers were treated with IFN-gamma (1 µg/ml) or cycloheximide (0.1 mg/ml) for 8 h; during the culture period the medium was changed every 8 h and collected at the points of interest. The supernatant media were centrifuged at 10 000 *g* for 10 min to remove cells and debris. The CA-125 concentration of the supernatant was determined using a sandwich solid phase radioimmunoassay (CA 12-5-K, Centocor, Malvern, PA). The cells in the tissue culture plate were detached and their number was assessed using an electronic particle counter (Coulter, Dunstable, UK) as recently described [8] to allow a correction of CA-125 release in relation to the number of cells.

CA-125 cytosolic levels

Ovarian carcinoma cells were cultured in 150 ml flasks and after pre-treatment with interferon-gamma or cycloheximide or the medium alone, respectively, they were detached using trypsin (0.05%)-EDTA (0.02%) in phosphate buffered saline (PBS). About 30×10^6 cells of each group were sonicated until more than 95% of the cells were disrupted. The homogenate was centrifuged at 100 000 *g* for 30 min. In the supernatant the concentration of CA-125 was determined using the radioimmunoassay mentioned above.

Table 1. CA-125 Expression in ovarian carcinoma cells

Cell line	Cytosol (U/mg)	Cytosol (µU/cell)	Membrane (cpm)	Medium [µU/(cell × 24 h)]	PDT (h)
OVCAR-3	560	76	1860	100	40
HTB-77	6	1	78	< 1	68
SKOV-6	390	75	930	75	38
SKOV-8	370	53	1530	95	85
SKUT-2	< 1	< 1	< 50	< 1	37
2774	< 1	< 1	< 50	< 1	37

Determination of CA-125 was performed 3 days after cell transfer and in a log growth phase. Results were shown as the mean of four measurements and the table represents one of three similar experiments.

Cell cycle analysis

The cells were inoculated into 150 ml tissue culture flasks and allowed to attach overnight. In order to obtain partial synchronisation, the cells were treated with hydroxyurea (20 nmol/l) for 2 days. The cell monolayers were then washed twice, and a complete medium was added. The cells of three flasks were detached immediately or after 24 or 48 h with the help of trypsin (0.05%)–EDTA (0.02%) in phosphate buffered saline (PBS). After centrifugation the cells were separated into two aliquots. In one part, the cytosolic CA-125 concentration was determined as described before, whereas the cells in the second part were stained with propidium iodide (50 µg/ml PBS containing 1% Triton X-100 for the purpose of DNA determination [9]. The resulting nuclear suspension was gently aspirated with a 22-gauge needle and filtrated through a 50 µm mesh screen to remove any clumps. Double and triple clumps constituted less than 3% of the total nuclear suspension. The fluorescence of 10 000 propidium iodide-stained nuclei was analysed with a FACS III (Becton and Dickinson, Sunnyvale, California). The number of cells either in the G_0/G_1, S, or the G_2/M-phase was calculated using the DNA Cell-Cycle Analysis Software (Becton and Dickinson, version C 5/87). The coefficient of variation was consistently lower than 4%.

RESULTS

The tumour marker CA-125 was expressed in four out of six cell lines tested (Table 1). In the three lines OVCAR-3, SKOV-6 and SKOV-8, high levels of CA-125 were detected in the cytosolic fraction, on the cell surface and in the culture medium. HTB-77 cells showed a lower cytosolic concentration and membrane presentation, and the release of CA-125 into the supernatant medium was not sufficient to be measured by our standard procedure. The ovarian and endometrial carcinoma cell lines 2774 and SKUT-2 did not express detectable amounts of CA-125 in any of the compartments analysed. The CA-125 values obtained for cytosol, medium and cell membrane showed a significant correlation (Spearman correlation coefficient for each comparison > 0.893, $P < 0.05$). We found that the amount of CA-125 shed into the culture medium in 24 h was in the same range as the amount measured in each cell (100–180%). This

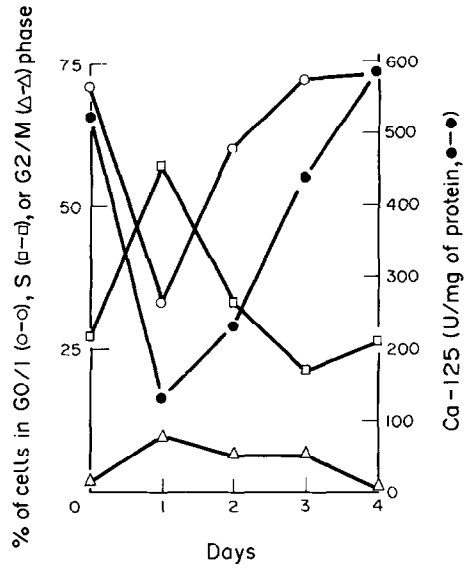

Fig. 1. CA-125 and cell cycle. OVCAR-3 cells were partially synchronised by pre-treatment with hydroxyurea (20 nmol/l). Then the CA-125 concentration in the cytosolic fraction was determined (●), and simultaneously, the distribution of cell nuclei in the different phases of the cycle (G_0/G_1 ○, S □, and G_2/M △) was estimated after staining them with propidium iodide as described in Materials and Methods. The figure shows one representative experiment out of three.

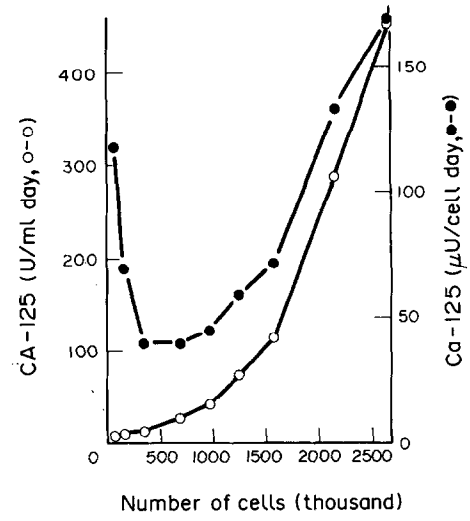

Fig. 2. Dependence of CA-125 shedding on cell density. Increasing numbers of OVCAR-3 cells were seeded and allowed to attach overnight. The culture medium was renewed and the cells were cultured for 24 h. The medium was collected and the CA-125 level determined; the OVCAR-3 cells were also detached and counted as described before. The CA-125 level shown is the mean value of the concentrations measured in six wells (○, left *y*-axis), and the mean release per cell and day of the same six wells (●, right *y*-axis). The coefficient of variation was consistently lower than 15% and is not shown for reasons of clarity. The figure shows one representative experiment out of three.

would imply that intracellular and membrane bound CA-125 has to be renewed by the cell every day.

The growth rate, as shown by the population doubling time was associated with neither CA-125 production nor shedding (Table 1). However, within a given cell line the level of CA-125 correlated with the percentage of cells in the G_0/G_1-phase of the mitotic cycle (Fig. 1). Partial synchronisation of the cells by means of hydroxyurea, which resulted in an accumulation of cells in the G_0/G_1-phase, was associated with a high concentration of the tumour marker. When the cells started to enter the S-phase, the concentration of CA-125 decreased ($P < 0.01$). Consequently, the great number of cells in the S-phase leads to a great number of dividing cells and subsequently to an increased cell density with an accumulation of cells in the G_0/G_1-phase and increasing levels of CA-125. This relation between growth rate and CA-125 level was also confirmed by another experiment. OVCAR-3 cells were cultured at varying cell densities for 24 h (Fig. 2). The CA-125 concentration in the medium strongly correlated with the cell number; statistical analysis, however, revealed no linear relationship. Moreover, when the amount of CA-125 released within 24 h was corrected for cell number, it was found to be dependent on cell density. Both low and high cell densities were associated with the highest CA-125 release ($P < 0.01$), whereas medium cell densities, which promote logarithmic growth rates, resulted in reduced CA-125 production per cell.

The results presented in Table 1 suggest that tumour cells are able to renew CA-125 within 24 h. In an attempt to prove this hypothesis, OVCAR-3 cells were treated with cycloheximide for 8 h to block protein biosynthesis (Fig. 3). Thereafter, the amounts of CA-125 expressed on the cell surface and shed into the culture medium were measured. For both compartments, this pre-treatment resulted in CA-125 levels reduced to about 15 and 55%, respectively. The maximum reduction occurred 3 days after incubation with cycloheximide; subsequently the cells recovered and CA-125 formation returned to its initial level on day 5 or 6.

Since the gene(s) of CA-125 have not yet been cloned, it is not possible to study their regulation directly. It is necessary to

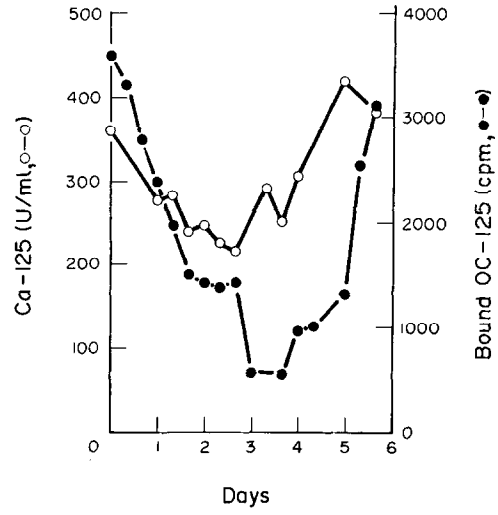

Fig. 3. Effects of cycloheximide on the expression and release of CA-125. OVCAR-3 cells were treated with cycloheximide (0.1 mg/ml) for 8 h. The culture medium was then renewed twice and the cells were fed with complete medium (time point 0). Over the following 6 days, the CA-125 surface expression (●, right *y*-axis) and the concentration in the medium (○, left *y*-axis) were measured as described in Materials and Methods. Each point represents the mean value of six cells measured. The coefficient of variation was consistently lower than 8% for surface expression and lower than 15% for the concentration in the medium and is not shown for reasons of clarity. The figure shows one of two similar experiments.

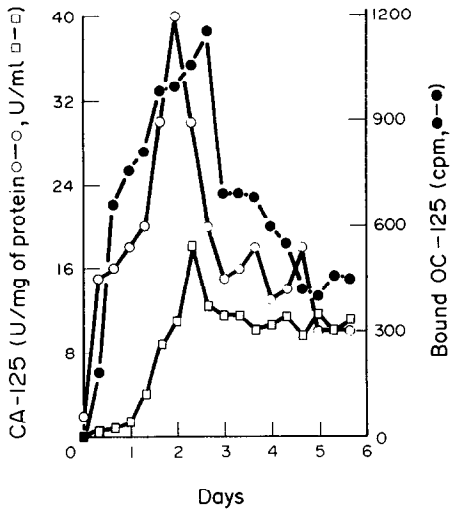

Fig. 4. Effects of interferon-gamma on CA-125 expression. HTB-77 cells were treated with interferon-gamma (1 ng/ml) for 8 h. The culture medium was then renewed twice and the cells were fed with complete medium (time point 0). Over the following 6 days, the CA-125 surface expression (●, right *y*-axis) and the concentration in the medium (□, left *y*-axis) and in cytosol (○, left *y*-axis) were measured as described in Materials and Methods. Each point represents the mean value of six wells measured. The coefficient of variation was consistently lower than 7% for surface expression and lower than 16% for the concentrations in the medium and in cytosol and is not shown for reasons of clarity. The figure shows one of two similar experiments.

introduce substances as auxiliary means which either suppress protein biosynthesis (e.g. cycloheximide) or induce antigen expression. As has recently been shown, interferons augment the expression of CA-125 in ovarian carcinoma cells [6]. Therefore, we were interested in comparing the kinetics of antigen induction by interferons with the kinetics of antigen inhibition by cycloheximide. HTB-77 cells were treated with interferon-gamma for 8 h and after replacing the culture medium, they were maintained without interferon supplementation (Fig. 4). Both in cytosol and on the surface membrane the expression of CA-125 showed a rapid increase during the first 2 days. Having reached the maximum values, the levels of cytosolic and membrane bound CA-125 declined rapidly, but did not reach the initial low value. After pre-treatment with interferon an increase in CA-125 concentration was found in the supernatant as well. However, this increase was significantly delayed. After the maximum level had been reached on days 2 or 3, the CA-125 concentration did not return to the initial level in this compartment either.

DISCUSSION

Bast *et al.* [2] first reported elevated serum levels of CA-125 in 83% of patients with surgically demonstrable epithelial ovarian cancer. Dissemination of tumour tissue outside the ovary is associated with an increase in serum CA-125 in more than 90% of cases, but when the tumour tissue is confined to the ovary, this tumour marker is elevated in 50% of cases only. CA-125 has become the most important tumour marker in the follow-up of patients with ovarian carcinoma, since an increase in CA-125 serum concentration has been shown to be associated with tumour progression. An elevated CA-125 serum level prior to second look laparotomy clearly indicates the presence of a tumour [4, 10]. CA-125 is elevated in only a small proportion of patients with microscopic disease, but in more than 70% of patients presenting with a tumour larger than 1 cm in diameter [5]. These observations clearly show that the most important factor governing this tumour marker must be the number of tumour cells. However, there is evidence that high CA-125 tissue levels are not necessarily associated with elevated CA-125 serum concentrations [5]. Therefore, we were interested in the mechanism regulating this tumour marker. Antigens recognised by the OC-125 were found to be shed very rapidly, and within approximately 24 h nearly the entire cellular content has to be renewed. This was demonstrated by comparing the amount of CA-125 in cytosol with the amount released in 24 h, and by blocking protein biosyntheis. A rapid turnover and a short half-life of a tumour marker are very important for clinical application, since they guarantee a rapid reflection of changes in tumour size and biology.

The influence of expression inducing substances was investigated with the aim of comparing the action of cycloheximide, a protein biosynthesis inhibitor, with the effect of naturally occurring agents. Up to now, only interferons have been shown to increase the CA-125 concentration in cytosol, on the surface membrane, and in the culture medium [8]. This was also confirmed by our study. However, it is very interesting that even in case of a pre-treatment period of 8 h, only a marked CA-125 peak occurred 2–3 days later. Subsequently, the levels of CA-125 dropped, but did not return to the initial values. The 'memory' observed could be explained by genes which are switched on by interferon and remain at least partially active, even when the starting agent has been removed. It is still unclear, whether endogenous interferon production (e.g. during a viral infection) may affect tumour marker production. Both the rapid decrease in CA-125 expression caused by cycloheximide and the rapid increase induced by interferon-gamma are consistent with a fast turnover of CA-125. Two suppressing factors have been described for ovarian carcinoma cells. Karlan *et al.* [11] observed decreased CA-125 expression in OVCA-433 ovarian carcinoma cells under the influence of dexamethasone; the transforming growth factor-β, a multifunctional peptide regulating several processes in ovarian cells, has recently been shown to reduce CA-125 expression in OVCAR-3 ovarian carcinoma cells [12]. For both substances similar kinetics of suppression have been described. However, the mechanism regulating CA-125 expression either by expression-inducing agents (interferons) or suppression substances (glucocorticoids and transforming growth factor-β) remains largely unknown.

It is very interesting that the shedding of CA-125 was influenced by cell division and cell cycle. Exponentially fast growing cells or cells with a high portion in the S- and G_2/M-phases showed suppressed CA-125 expression and shedding. The shedding of CA-125 in terms of U/cell was found to be dependent on cell density. At a high cell density (plateau phase), the growth fraction decreases because of reduced cell spreading, depletion of nutrients and growth factors in the medium, and contact inhibition [13]. On the other hand, at a low cell density (lag phase) the initiation of proliferation is delayed, because the adaptation of the cells to culture conditions is prolonged by a lack of autocrine growth factors. At a medium cell density, the highest growth fraction is achieved (log phase), since the cells have both sufficient growth factors and enough space for cell division [13]. During this phase CA-125 shedding was markedly decreased, which is in contrast to the behaviour of other glycoproteins. As regards plasma membrane (glyco)proteins, recent studies indicate that there is an increased turnover in growing

cells, which may result in an increased release into the medium [14]. In cultured prostate cancer cells the levels of tumour-associated antigens, such as the prostate-specific acid phosphatase or epithelial membrane antigen, also increase during the G_1 phase, remain stable during the G_2 phase and drop during or immediately after cytokinesis [15]. The different behaviour of glycoproteins might be explained by the as yet unclear function of CA-125, which is possibly involved in the homeostasis of resting cells.

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Biological Characterisation of Primary and Metachronous Lesions in Breast Cancer Patients

Rosella Silvestrini, Barbara Valentinis, Maria Grazia Daidone, Giovanni Di Fronzo, Danila Coradini and Bruno Salvadori

Proliferative activity, evaluated as [^{3}H]thymidine labelling index ([^{3}H]dT LI), and hormone receptors were determined on 97 primary breast cancers and on metachronous lesions from the same patient. Overall, the [^{3}H]dT LI of metachronous lesions was significantly higher than that of the primary tumour ($P = 0.003$). Hormone receptor profiles of the two lesions were similar in about 75% of the cases; disagreements were mainly due to a disappearance of hormone receptors in metachronous lesions. In contralateral tumours, [^{3}H]dT LI and hormone receptors were unrelated to those of the relative primary lesion. In this series of relapsing patients, [^{3}H]dT LI was unrelated to hormone receptor status in the primary tumour, but it was higher in the metachronous lesions from patients with hormone receptor-negative primary tumours. For patients given no systemic therapy between surgery and relapse, the time to develop local–regional recurrences or contralateral tumours was inversely related to the [^{3}H]dT LI of the metachronous lesions.
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INTRODUCTION

CANCER IS characterised by a marked biological heterogeneity not only among tumours of different patients, but also among the different synchronous or metachronous lesions of the individual patient. In fact, cell clones with a different biological profile in terms of karyotype, antigenicity, metastatic potential, biochemical properties, hormone receptor status, growth behaviour, chemo- and radiosensitivity may co-exist within the same tumour as a balance between natural or induced selective pressure [1–4]. Moreover, with progression of the disease, the overgrowth of