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Soluble Intercellular Adhesion Molecule-1 (ICAM-1) is Released into the Serum and Ascites of Human Ovarian Carcinoma Patients and in Nude Mice Bearing Tumour Xenografts

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We have demonstrated that patients with ovarian carcinoma have higher levels of soluble intercellular adhesion molecule-1 (ICAM-1) in their serum and ascitic fluids than serum from normal individuals and non-neoplastic gynaecological disease or ascites from patients with cirrhosis. In order to investigate the source of the ICAM-1, and to study the mechanisms which regulate ICAM-1 relase in ovarian carcinoma, we have employed the nude mouse model system. Three different human ovarian carcinoma (HOC) cell lines were grown as ascitic tumours in the peritoneal cavity of nude mice. HOC xenografts harvested from nude mice expressed comparable levels of ICAM-1 on their cell surface. Human ICAM-1 was detected, with a species-specific ELISA, in serum and ascitic fluid of tumour-bearing mice, confirming that the tumours were the source of the ICAM-1. The three HOC xenografts showed different levels of ICAM-1 release, but within each xenograft model the level of ICAM-1 in serum and ascitic fluid correlated with the tumour burden. The level of ICAM-1 released by the HOC xenografts could be increased by *in vivo* treatment with interferon gamma (IFNγ). Interleukin 1 (IL-1), tumour necrosis factor (TNF) and IFNγ increased the cell surface expression of ICAM-1 and caused the release of soluble ICAM-1 from HOC cells established *in vitro*. The nude mouse provides a useful system in which to study the effects of modulating ICAM-1 release on the progression of ovarian carcinoma and suggests that measuring ICAM-1 levels in the blood or ascites of patients may provide an indication of tumour burden.

Key words: ovarian carcinoma, intercellular adhesion molecule-1, interferon, nude mouse
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INTRODUCTION

CELLULAR ADHESION molecules have been implicated in homotypic and heterotypic cell interactions. Intercellular adhesion molecule-1 (ICAM-1) (CD-54), an adhesion ligand for β2 integrins LFA-1(CD11a/CD18) and MAC-1(CD11b/CD18), has been shown on various cell types, including vascular endothelial cells and leucocytes, and its expression can be modulated by cytokines [1]. ICAM-1 plays a role in inflammatory and immune responses [2, 3], and it has been implicated in the migration of tumour and normal cells. While ICAM-1 has been extensively studied on melanomas, where its expression has been associated with tumour progression and metastasis [4, 5], restricted examples of other tumour types expressing ICAM-1 have been reported [6].

Recently soluble forms of circulating sICAM-1 have been described in serum of normal donors, with elevated levels in serum of patients with various diseases [7–10]. Circulating sICAM-1 has been shown in blood of cancer patients, with higher levels being associated with malignant disease [11–13]. We have shown that human melanoma cells release sICAM-1, and high levels of sICAM-1 were found in the serum of nude mice bearing human melanoma [14].

In advanced human ovarian carcinoma, tumour cells spread throughout the peritoneal cavity [15]. We have previously

established and characterised intraperitoneal human ovarian carcinoma xenograft models in nude mice [16]. Human ovarian carcinomas (HOC) that produce ascites and spread in intra-abdominal organs of nude mice mimic clinical disease, and provide an ideal model to study tumour malignancy.

In this study, we investigated the expression and release of ICAM-1 from human ovarian carcinomas. Using the intraperitoneal (i.p.) xenograft model, we show that the level of sICAM-1 in serum and ascites is associated with tumour burden, and can be modulated by cytokines.

MATERIAL AND METHODS

Mice and tumour lines

Female NCr nu/nu nude mice, 6–8 weeks old, were obtained from the National Cancer Institute, Animal Program, Frederick, MD, U.S.A. Mice were maintained throughout the experiments under specific-pathogen-free conditions as described previously [16].

The human ovarian carcinoma xenografts, HOC8, derived from pleural effusion, and HOC10 and HOC22, obtained from ascites of patients with ovarian carcinomas, were established and maintained as ascites in nude mice as described previously [16, 17]. HOC cell suspensions grew in the peritoneal cavity of all the injected mice, producing ascites and infiltration of the peritoneal organs [16, 17]. The HOC10-TC line was established from HOC10 ascites, and was cultured as a monolayer in RPMI with 10% FCS. Cultures were used at passage 20–25.

Reagents and antibodies

Antibodies specific for human ICAM-1 (BBIG-I1 and BBIG-I2) were obtained from British Biotechnology Products, Abingdon, Oxfordshire. The following cytokines were provided from the indicated companies: recombinant human interleukin-1β (IL-1) (specific activity, 3×10^8 units/mg) from Sclavo, Italy; tumour necrosis factor α (TNF) (specific activity, 8.1×10^6 units/mg) from Basf-Knoll, Germany; interferon gamma (IFNγ) (specific activity, 20×10^6 units/mg) and recombinant rat IFNγ (specific activity, 10×10^6 units/mg) from Roussel UCLAF, France. Cytokines provided as lyophilised preparations, were diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and stored in aliquots at −80°C until used. For injections, cytokines were diluted in pyrogen-free sterile PBS.

Patients

Blood samples and ascitic fluids were collected from untreated patients with histologically confirmed epithelial ovarian carcinoma admitted to the Department of Obstetrics and Gynaecology, S. Gerardo Hospital, Monza, Italy. All patients had cancer classified as International Federation of Gynaecologists and Obstetricians stage III and IV. Sera were obtained by blood coagulation, and ascitic fluid was collected by paracentesis, centrifuged and cell-free supernatants used in the assay. Serum was also obtained from patients with non-neoplastic gynaecological disease and ascitic fluid from patients with chronic cirrhosis. Normal blood serum was obtained from female healthy volunteers. All samples were stored at −80°C until used in the assay.

ICAM-1 from cultured HOC

HOC10-TC cells harvested by exposure to 0.25 trypsin–0.02 EDTA solution, were washed twice and seeded (2×10^5 cells) in 6-well culture plates in 2 ml RPMI medium with 10% FCS (test medium). After 24 h, monolayers were washed and 2 ml test medium with or without cytokines (500 U/ml) were added. After 72 h, supernatants from treated and untreated wells were collected, centrifuged and frozen at −80°C. To evaluate cell-associated ICAM-1, the cell monolayers were washed twice, subjected to two rounds of freeze/thawing, mechanically removed, sonicated to yield a cell lysate and frozen at −80°C until analysis. Protein content of cell extract was determined with the Bradford procedure (Bio-Rad Protein Assay, Bio-Rad, California, U.S.A.). Tumour cells were also harvested from separate wells using versene and processed for FACS analysis, as described below.

ICAM-1 from HOC in nude mice

HOC8, HOC22, HOC10 were injected i.p. as a 10×10^6 cell suspension in nude mice. Sera and ascites were obtained at different times after tumour cell injection as specified in the results (three mice for each time point). The number of viable cells in the ascites was counted by trypan blue exclusion, and used as an indication of tumour burden. Blood was obtained by intracardiac puncture from anaesthetised mice, and ascites were harvested with 3 ml of 0.9% NaCl. Coagulated blood and ascites were centrifuged at 3000 rpm for 10 min. Serum and ascitic fluid were collected, frozen and maintained at −80°C before analysis. Blood and peritoneal washes were obtained from non-tumour-bearing mice and processed in the same way. To evaluate the effect of IFNγ, mice were given i.p. injections of 2×10^5 units IFNγ in 0.2 ml solution once daily for 4 consecutive days, starting 15 days after HOC transplantation. Control mice received the same amount of vehicle. Two hours after the last treatment, blood and peritoneal washes were harvested and processed as described above. Aliquots of tumour cells from nude mice (5×10^6 in 1 ml PBS) were also processed for FACS analysis to evaluate ICAM-1 expression and lysated to evaluate cell-associated ICAM-1.

ICAM-1 expression

HOC cells harvested from nude mouse ascites or from cultures were washed twice in PBS and processed for FACS analysis as described before [14]. Briefly, after incubation with the appropriate primary anti-ICAM-1 antibody in PBS with 2% human serum albumin (HSA) for 30 min at 4°C, cells were washed and incubated with an affinity-purified FITC-labelled goat antimouse immunoglobulin antiserum for 30 min at 4°C (Tecno Genetics, Italy). The expression of ICAM-1 was determined by indirect immunofluorescence using FACS-STAR-Plus (Beckton-Dickinson). Results were expressed as percentage positive cells and mean channel fluorescence intensity.

Assay of soluble ICAM

Levels of soluble human ICAM-1 were measured using a commercial ELISA kit (British Biotechnology Products).

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Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC council directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH guide for the care and use of laboratory animals, NIH publication n. 85-23, 1985).
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Statistical analysis

The levels of ICAM-1 in the serum of healthy controls were compared with those in ovarian carcinoma patients, and the levels in ovarian carcinoma ascites with cirrhosis ascites, using Student's *t*-test.

RESULTS

Circulating ICAM-1 in ovarian carcinoma patients

Figure 1 shows that sICAM-1 is present in sera from healthy donors at levels (median 181.9 ng/ml, range 85.3–274.1) that are consistent with previous reports [11–13] reviewed in [10]. Elevated levels of sICAM-1 were detected in the serum and ascitic fluid of patients with ovarian carcinoma: median concentration was 305 ng/ml (range 146.3–594.8) and 335 ng/ml (range 128.5–587.8), respectively, in serum and ascitic fluid (Figure 1). sICAM-1 was significantly elevated in the serum of patients with ovarian carcinoma ($P = 0.001$), while the level of sICAM-1 in sera of patients with non-neoplastic gynaecological disease was not significantly different from controls (Figure 1). The level of sICAM-1 in the ovarian carcinoma ascites was also higher than in samples of cirrhosis ascites (median 130.44 mg/ml, range 26.8–384, $P = 0.001$).

sICAM-1 secretion by ovarian carcinomas in nude mice

sICAM-1 was found in the serum and ascitic fluid of nude mice bearing each of the three HOC xenografts in the peritoneal cavity. The number of cells in the ascites (an indication of tumour burden) of nude mice increased with time from tumour injection (Figure 2). The level of sICAM-1 in sera and ascites increased with time after tumour transplant, showing a direct correlation with the tumour burden. At comparable times, the sICAM-1 levels in serum were lower than in ascitic fluid for HOC10 and HOC22, while no significant differences were observed between sICAM-1 levels in serum and ascitic fluid of mice bearing HOC8. Serum and peritoneal washes from control vehicle injected mice and tumour-free mice did not contain detectable levels of sICAM-1 (data not shown). The level of sICAM-1 released at similar tumour burdens differed among the

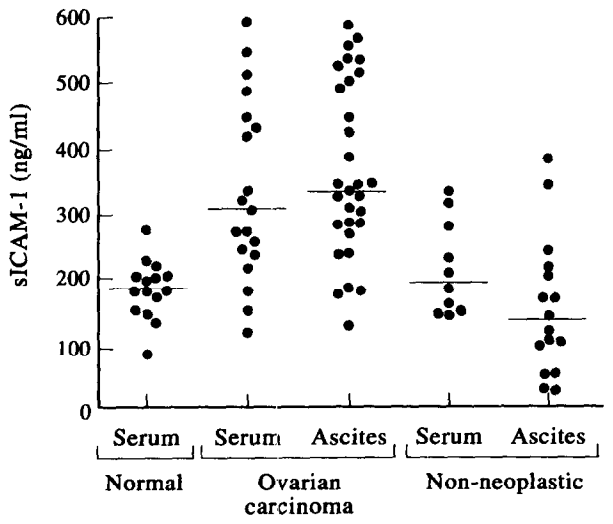

Figure 1. ICAM-1 secretion in patients with ovarian carcinoma. Levels of sICAM-1 (ng/ml) in sera from normal healthy donors ($n = 15$), sera ($n = 19$) and ascitic fluid ($n = 30$) from ovarian carcinoma patients, sera ($n = 10$) from patients with non-neoplastic, gynaecological disease and ascitic fluid ($n = 16$) from patients with cirrhosis. Horizontal bars represent the median values.

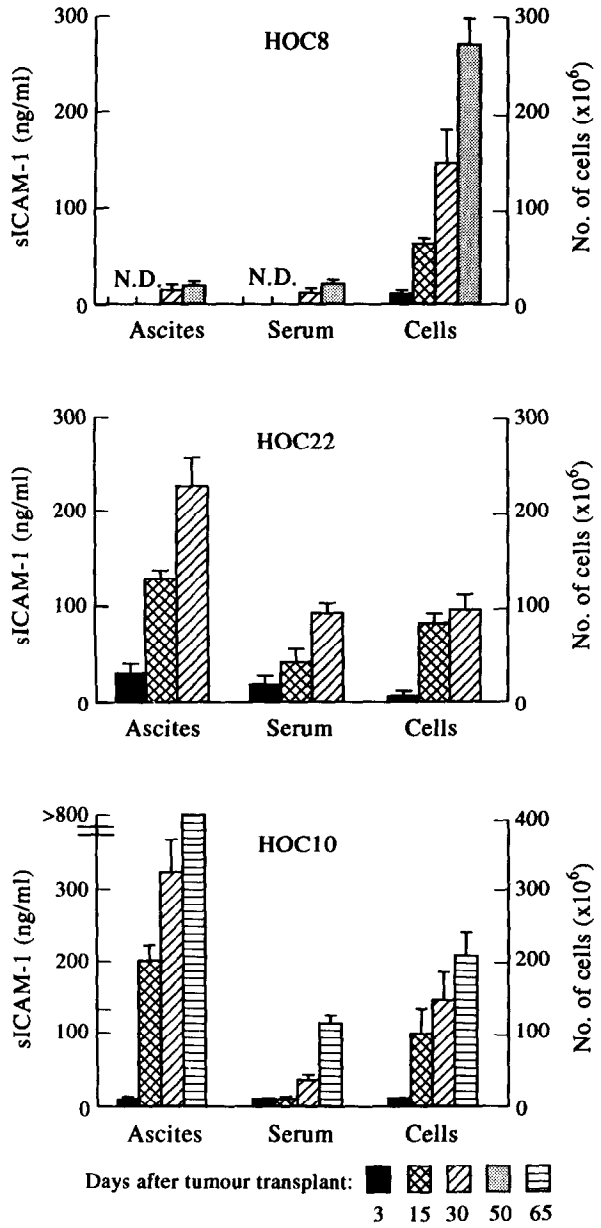

Figure 2. ICAM-1 secretion in nude mice bearing HOC xenografts. Nude mice were injected i.p. with HOC8, HOC22, HOC10 xenografts. sICAM-1 was measured in sera and ascitic fluid at different times after tumour injection. Number of cells is the number harvested from the peritoneal cavity at different times. Results are the mean ± S.D. of three mice per group. N.D. = below the limit of detection.

three xenografts, with HOC10 > HOC22 ⩾ HOC8. This difference prompted us to investigate the expression of ICAM-1 on the HOC cell surface. ICAM-1 was highly expressed on the cell surface of the three HOC xenografts harvested from ascites of nude mice. The percentage of ICAM-1 positive cells, 30 days after tumour transplant, was 77, 60 and 88%, respectively, for HOC8, HOC22 and HOC10 with a mean fluorescence intensity of 544, 576 and 574 (data not shown).

Effect of IFNγ on ICAM-1 release from ovarian carcinoma xenograft

The treatment of mice with hu IFNγ for 4 days, 15 days after tumour inoculation, caused a decrease in tumour burden as

measured by the number of cells in the peritoneal washes (data not shown). However, the levels of sICAM-1 in serum and ascites fluid were augmented in mice treated with hu IFNγ (Figure 3). To verify that this augmentation was associated with up-regulation of ICAM-1 on tumour cells after IFNγ treatment, we measured the expression of ICAM-1 on cells harvested from ascites of treated nude mice. Table 1 confirms that HOC8 cells do not release detectable levels of sICAM-1 into serum and ascitic fluid of untreated mice, although cell associated ICAM-1 was present (cell surface/cell associated). After treatment with hu IFNγ, sICAM-1 was found in serum and ascites of nude mice, and ICAM-1 expression was increased on HOC8 cells as measured by FACS analysis and by extraction of cell associated ICAM-1 (Table 1). These findings show that ICAM-1 expression is modulated by IFNγ, and suggest a role of IFNγ in ICAM-1 release. Treatment with rat IFNγ did not affect the expression of ICAM-1 on HOC8 cells, and gave low levels of sICAM-1 release into serum and ascitic fluids of nude mice (Table 1).

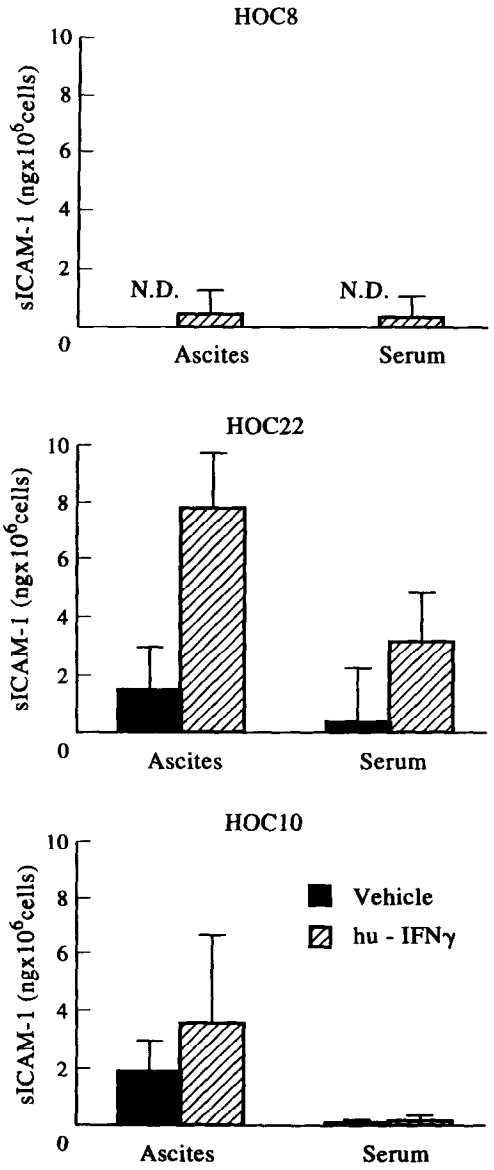

Figure 3. ICAM-1 secretion in nude mice bearing HOC xenografts after IFNγ treatment. Nude mice were injected i.p. with HOC8, HOC22 and HOC10 xenografts and 15 days later treated once daily for 4 days with hu IFNγ. Control mice received the same amount of vehicle. At the end of treatment, sICAM-1 was measured in sera and ascitic fluid. Results are expressed as ng per 10^6 viable cells harvested from the peritoneal cavity. Results are the mean ± S.D. of three mice per group. N.D. = below the limit of detection.

sICAM-1 expression and secretion by cultured HOC10-TC cells

To further confirm that the expression of ICAM-1 was a property of ovarian carcinoma cells, the expression of ICAM-1 was studied on HOC10-TC, a line established *in vitro* from HOC10 ascites. Table 2 shows that ICAM-1 was maintained on cell surfaces of HOC10-TC cultured *in vitro*. HOC10-TC cells, stimulated with IL-1, IFNγ and TNF, expressed higher levels of ICAM-1, as measured by FACS analysis and by extraction of cell associated ICAM-1. Low levels of ICAM-1 were released into culture supernatants of unstimulated HOC10-TC cells. The treatment with IL-1, TNF and IFNγ induced the release of significant amounts of sICAM-1.

DISCUSSION

In this report, we show that elevated levels of sICAM-1 are present in ascites and serum of ovarian carcinoma patients, and similarly, sICAM-1 is present in sera and ascites of nude mice bearing HOC in the peritoneal cavity. Similar levels of sICAM-1 to those we detect have been reported in sera of normal subjects and cancer patients, including those with ovarian carcinomas, but the source of sICAM-1, host cells or tumour cells, was not identified [11–13]. Using an ELISA, which is specific for human ICAM-1 and not murine ICAM-1, we have shown previously that human melanoma cells transplanted in nude mice are the source of sICAM-1 release into the serum [14]. Here, the use of the HOC xenograft model allowed us to demonstrate that human ovarian tumour cells release sICAM-1, and that levels of sICAM-1 in ascites and serum are related to tumour burden in mice. All three HOC lines express cell surface ICAM-1; however, significant differences in the levels of sICAM-1 released into serum and ascitic fluid were observed among the lines. Similar differences were observed measuring sICAM-1 level in the supernatants of the three HOC xenografts after 24 h in culture (data not shown). Specifically, the HOC8 tumour secreted low levels of sICAM-1 even in mice with advanced stage tumours, although high levels of cell-associated sICAM-1 were always measured by freeze-thawing HOC8 cells derived from ascites (data not shown and Table 1). This suggests different sensitivity to the mechanisms which regulate the release of ICAM-1 among the HOC lines. In addition, the fact that IFNγ treatment induced or increased the secretion of sICAM-1 indicates the importance of cytokines in this phenomenon.

We have taken the number of cells in ascites as an indication of tumour burden in nude mice, which is likely to underestimate the total tumour mass within the peritoneal cavity. However, while comparison of sICAM-1 between the three HOC lines could be affected by this evaluation, results in Figure 2 show a clear increase of sICAM-1 levels in serum and ascitic fluid with time of tumour growth within each HOC xenograft model. These results support the importance of sICAM-1 as a marker of progressive cancer. sICAM-1 is present in serum from normal individuals [9], but we have found that the ovarian carcinoma patients examined in this study had serum levels of sICAM-1 higher than healthy donors. In addition, we found high levels of sICAM-1 in ascitic fluid of patients. It is noteworthy that ascitic fluid, obtained from patients with chronic cirrhosis and serum obtained from patients with non-neoplastic disease, had levels of ICAM-1 below the median level found in cancer patients and

Table 1. ICAM-1 expression and release by HOC8 xenografts following in vivo *treatment with IFNγ*

Stimulus*	sICAM-1 (ng/ml)†		ICAM-1 expression‡	
	Ascites	Serum	Mean fluorescence (% positive cells)	Cell-associated (ng/mg)
Vehicle	N.D.	N.D.	548 (41%)	3.6
r Hu-IFN γ	18.8 ± 3.3	25.2 ± 4.1	711 (80%)	10.3
r Rat-IFN γ	4.8 ± 4.2	3.9 ± 3.4	487 (47%)	3.5

*Nude mice bearing HOC8 xenografts in the peritoneal cavity were treated with IFNγ as described in Figure 3.
†sICAM-1 in serum and ascitic fluid of tumour-bearing mice at the end of treatment. Results are the mean of triplicates ±S.D.
‡Cells from ascites were harvested at the end of treatment and ICAM-1 expression measured by FACS analysis (mean fluorescence) and by release in cell extracts (cell-associated).
N.D. = below the limit of detection.

Table 2. ICAM-1 expression and release by HOC10-TC established in vitro

Stimulus*	Mean fluorescence (% positive cells)†	sICAM-1‡	
		Secreted (ng/ml)	Associated (ng/mg)
Medium	167 (77)	0.08 ± 0.01	3.6 ± 0.2
IL-1	510 (96)	1.48 ± 0.04	18 ± 0.08
TNF	363 (91)	0.67 ± 0.01	5.2 ± 0.3
IFNγ	397 (95)	1.5 ± 0.01	32 ± 0.09

*Tumour cells were cultured with or without stimulus for 72 h.
†Tumour cells were harvested at the end of treatment and ICAM-1-expression measured by FACS analysis.
‡sICAM-1 was measured in supernatant (secreted) and cell extracts (associated). Results are the mean of triplicates ± S.D.

at a similar level of that of healthy donors. Further studies are necessary to evaluate the relevance of these findings in patients at different stages of disease or under therapeutic treatment.

The expression and shedding of ICAM-1 from tumour cells can be stimulated by cytokines [14, 18, 19]. Treatment of ovarian carcinoma patients with IFNγ causes increases in major histocompatibility antigen expression in tumour cells, and modulation of cytokine production by host cells [20]. It is therefore possible that the shedding of ICAM-1 in human cancers is also indirectly mediated by host-related cytokines, that in turn stimulate ICAM-1 expression and release. We show that sICAM-1 is not detectable in serum and ascitic fluid of nude mice bearing 19 day HOC8 xenografts, although after treatment with hu IFNγ, both serum and ascites contain sICAM-1. Since hu IFNγ has no biological activity on murine cells, the augmented release of ICAM-1 from the tumour is probably due to a direct effect on HOC cells. Rat IFNγ, which is active on murine cells [21], also caused detectable shedding of ICAM in nude mice bearing HOC xenografts. We also observed increased cell surface/cell associated ICAM-1 on HOC cells harvested from ascites of mice treated with hu IFNγ, but not those treated with rat IFNγ (Table 1). Other cytokines that cross species could also be responsible for sICAM-1 secretion. Indeed, we found that HOC8, HOC10 and HOC22 harvested from ascites and cultured for 24 or 72 h in the presence of IL-1, TNF and IFNγ release higher levels of sICAM-1 than untreated cells (data not shown).

That the expression and release of ICAM-1 can be a characteristic of carcinoma cells of ovarian origin is demonstrated by the results with the HOC10-TC line, established *in vitro* from HOC10. However, ICAM-1 expression on HOC10-TC seems somewhat reduced, suggesting a nude mouse environment effect on ICAM-1 expression. This is in contrast to a previous report that showed down-regulation of ICAM-1 on melanoma xenografts compared with the equivalent cell line *in vitro* [22].

The expression and shedding of ICAM-1 may have profound implications in tumour progression, ascites formation and metastasis. The importance of adhesion molecules, including ICAM-1 in mediating tumour recognition by monocytes, T-cells and natural killer cells has become evident [23]. Administration of cytokines, such as IFNγ and TNF, can increase ICAM-1 expression on tumour cells and increase recognition and susceptibility to killing by host cells [18, 24, 25]. Conversely, shedding of adhesion molecules by tumour cells may allow their escape from immunosurveillance by decreasing ICAM-1 mediated recognition. Cytokines that can induce this phenomenon [14, 19, 26] could therefore act in a detrimental way. However, in a limited clinical trial, IFNγ given i.p. has appreciable antitumour activity in ovarian carcinoma patients with minimal

residual disease resistant to chemotherapy [27]. Antitumour activity under these conditions has been associated with modulation of *in situ* effector cells [27]. It is therefore likely that the effects of IFNγ reflect a balance of its positive immunomodulatory effects and potential negative effects due to ICAM-1 release. The results reported provide a preclinical basis to explore the value of sICAM-1 for the monitoring of i.p. immunotherapy of ovarian carcinoma.

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