Giant Syncytia and Virus-Like Particles in Ovarian Carcinoma Cells Isolated from Ascites Fluid

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Ovarian cancer cells were isolated from ascites fluid of 30 different patients diagnosed with cystadenocarcinoma of ovaries. Large colonies of malignant ASC cells were observed during the first week of cell growth in vitro. Colony formation was followed by fusion of cells and formation of large multinucleated and highly vacuolated syncyti. In contrast, cells isolated from the ascites fluid produced by patients with benign mucinous cystadenoma of ovaries did not form syncyti. Nonmalignant Brenner tumor cells, isolated from the ascites fluid, also did not form syncyti. Syncyti, but not the nonmalignant tumor cells, were immunofluorescence stained with an anti-human immunodeficiency virus type 1 (HIV-1) gp120 monoclonal antibody (MAb) and MAb RAK-Brl. Both MAbs recognized cancer-associated antigens RAK (for Rakowicz markers) p120, p42, and p25. Exposure of ASC cells to either the anti-HIV-1 gp120 MAb or MAb RAK-Brl inhibited syncytia formation. PCR with HIV-1 Env-derived primers revealed DNA sequences with over 90% homology to HIV-1 gp41 in syncyti and in ovarian cancer cells but not in normal ovary cells. Electron microscopic analysis revealed viral particles, hexagonal in shape (90 nm in diameter), with a dense central core surrounded by an inner translucent capsid and dense outer shell with projections. Negative staining detected membrane-covered particles (100 to 110 nm in diameter) in the cell culture medium. Incubation of normal breast cells with viral particles resulted in drastic morphological changes and syncytium formation by the transformed breast cells. The cytopathic effects of the identified virus resembled those of spumaviruses, which, in addition to their epitopic and genetic homology to HIV-1, might suggest a common phylogeny.

Ovarian carcinoma is the most common cause of death from gynecological malignant neoplasm. The typically advanced stage of presentation and aggressive nature of ovarian cancer result in an overall 5-year survival rate of less than 40% of the patients (3, 16, 27). The recent progress in characterization and sequencing of the breast cancer (BRCA) genes (15) is believed to have a critical impact on diagnosis of the inherited forms of ovarian cancer. Since an inherited ovarian cancer is rare (2), the majority of women with BRCA gene-independent cancer will be diagnosed late. Further understanding of the etiology of ovarian cancer would significantly improve diagnosis and decrease the mortality of female patients.

We have recently identified a new family of cancer antigens that are selectively associated with breast and gynecological cancer in women (19–21, 24, 25) and prostate cancer in men (17), but which are absent in normal tissues. These antigens, named after investigator Rakowicz markers (or, briefly, markers RAK) p120, p42, and p25, exhibit biochemical, immunological, and genetic homology to the proteins of human immunodeficiency virus type 1 (HIV-1) (17–21, 24, 25). One of the antigens, antigen RAK (M₄, 160,000), is detectable in the blood of over 90% of ovarian, cervical, and breast cancer patients, but in only 25% of healthy women with a family history of breast or gynecological cancer and 13% of healthy women with no relatives affected by female cancer (23, 25). The clear association of the antigen RAK with cancers of the reproductive system suggests that it may represent a breakthrough in the early diagnosis of these malignancies.

PCR with HIV-1 gp41-derived primers revealed breast and prostate cancer-associated DNA sequences with over 90% homology to HIV-1 (17, 18, 24). These novel DNA sequences have been deposited in the GenBank as cancer-associated gene RAK alpha. It is noteworthy that HIV-1-related DNA sequences are absent in normal tissues of cancer patients, which suggests an exogenous (viral) origin. The viral origin of markers RAK was recently confirmed by finding breast cancer and cervical cancer-associated viral particles, immunoreactive with a monoclonal antibody (MAb) specific for HIV-1 gp120 (MAb 5023) (18). Moreover, antisense oligonucleotides complementary to the HIV-1-like sequences inhibited reverse transcriptase activity in breast and cervical cancer cells and inhibited proliferation of these cells (18).

Over 90% of ovarian cancer patients also express antigen RAK in the blood and antigens p120, p45, and p24 in the malignant tissue (20, 25). The homology of ovarian cancer antigens with HIV proteins strongly suggests that an unknown retrovirus might also play a role in malignancies of the ovary. In the present report, both HIV-1-like sequences and viral particles were detected in ovarian cancer cells obtained from malignant ascites fluid produced by patients with ovarian cystadenocarcinomas. The cytopathic effects observed in cancer cells isolated from ascites fluid resembled the cytopathic effects caused by spumaviruses.

Spumaviruses, oncoviruses, and lentiviruses belong to the same family, Retroviridae (14). In contrast to the oncoviruses, spumaviruses have not been directly implicated in the development of neoplasia, although it was reported that, under certain conditions, the human spumavirus is capable of inducing transformation of human fibroblasts (11). The studies reported in this communication suggest that a virus genetically related to HIV-1, which induces effects resembling those induced by spumaviruses, may be present in a latent form in ovarian cancer cells.
MATERIALS AND METHODS

Cell culture. Ascitic fluid was obtained from 30 different patients with cystadenocarcinoma of the ovary. The control ascitic fluid was collected from five patients with mucinous cystadenoma and one patient with a Brenner tumor, which are nonmalignant tumors of the ovary. All samples were collected without patients' identifiers, according to the University of Nebraska Medical Center Epilepsy Cancer Center IRB-approved protocols. Experiments were done in a blind fashion. Ascitic cells were centrifuged at 2,000 × g for 5 min, and 50% of the supernatant was replaced with the Eagle's medium–L15 (3:1)–10% fetal bovine serum as the cell culture medium (GIBCO BRL). Cells were mixed with the medium and transferred to the cell culture flasks. The medium was replaced after 1, 2, and 3. Starting at day 6, cells were treated with 0.1% trypsin once a week and passaged into new culture flasks. Cells obtained from each of 25 cystadencarcinoma patients (ASC I to V, VIII to XV, XIX, and XX to XXIX), the mucinous cystadenoma patients (MC), and from the Brenner tumor patient (BREN I) were grown at different times without any possibility of a cross-contamination. ASC VI was grown in the presence of ASC VII, ASC XVI cystadenocarcinoma patients (ASC I to V, VIII to XV, XIX, and XX to XXIX), from the mucinous cystadenoma patients (MC), and from the Brenner tumor patient (BREN I) were grown at different times without any possibility of a cross-contamination. ASC VI was grown in the presence of ASC VII, ASC XVI was grown in the presence of ASC XVII, and ASC XXX was grown in the presence of the aging ASC XXIX cells. Cells were grown in a CO2 incubator in the absence of other cell lines.

Cell line. Ovarian carcinoma cell line CaOV-3 was purchased from the American Type Culture Collection and was grown in the same medium as ASC cells (described above). Normal OVCAR cell line 633.Hs578Bst (American Type Culture Collection) was grown in a normal cell medium provided by the American Type Culture Collection.

MAbs. Epitope-specific anti-HIV-1 gp120 MAb 5023 was obtained from DuPont (NEA-9301). The MAb RAK-B1 was developed previously as a monoclonal antibody against gp120 gp160 (18, 24). Both the anti-HIV-1 gp120 MAb and MAb RAK-B1 bind to the same cancer antigens and recognize similar epitopes (17–21, 24, 25). Both MAbs also recognize gp160 and gp120 of HIV-1 (18). The control anti-HIV-1 gp120 MAB 5023, which does not recognize cancer antigens (19), was also obtained from DuPont (NEA-9301). The control anticancer MAB C63.3 was raised against squamous cell-associated antigens (22) by our laboratory.

Microscopic analysis. A TMS Nikon microscope with a phase-contrast mechanism and the attached camera system was used to analyze syncytium formation. Cells were photographed in the cell culture medium.

Immunofluorescence staining. For immunofluorescence staining, cells were incubated for 24 h at 37°C either with MAB 5023 (100 ng/ml) or MAB RAK-B1 or with the control MAb 5023 or MAB C63.3, as described previously (19). After incubation, cells were washed with phosphate-buffered saline (PBS), fixed with 50% and 100% ethanol, and incubated for 1 h at 37°C with fluorescein-labeled goat anti-mouse immunoglobulin G (IgG). A Labophot-2 Nikon microscope was used to analyze the cells.

Electron microscopic analysis. Cell culture supernatant was clarified by low-speed centrifugation at 2,000 × g for 10 min. Viral particles were ultracentrifuged at 110,000 × g for 1.5 h in a type 19 Beckman tube. The pellet was suspended in PBS and placed on 300-mesh copper grids coated with Formvar and carbon (Structure Proc., Inc.). Samples were stained with uranyl acetate and examined with a Hitachi HU-12A transmission electron microscope. This service was performed by Advanced Biotechnology, Inc.

Cell fractionation. Fractionation of cells and tissues was performed as described before (19, 21). Briefly, cancer and normal tissue samples were homogenized in 0.35 M sucrose–10 mM KCl–1.5 mM MgCl2–10 mM Tris-HCl (pH 7.6)–0.12% Triton X-100–12 mM 2-mercaptoethanol and centrifuged at 600 × g for 10 min. With the cell lines and cell cultures, cells were scraped from the plastic, washed three times with PBS, and homogenized as described above (4 × 106 cells/1 ml).

Western blotting. For Western blotting, proteins were separated in 10% polyacrylamide gel with sodium dodecyl sulfate as described previously (24). Approximately 100 to 120 μg of protein was loaded on each lane. The blotting of proteins from the polyacrylamide gel to the polyvinylidene difluoride (PVDF) membrane was performed in a buffer containing 192 mM glycine, 25 mM Tris-HCl (pH 8.6), and 10% methanol. Transfer onto the PVDF membrane was performed at 50 V overnight. Membranes were washed with water, followed by Tris-glycine buffer. Filters were incubated with 1% bovine serum albumin for 1 h at 4°C, incubated with MAB RAK-B1 (0.1 μg/ml) or MAB 5023 (1 μg/ml), washed with Tris-glycine buffer, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h. After being washed with the Tris-glycine buffer, membranes were incubated with 0.1% i-naphthol-phosphate and Fast Red in a mixture of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl2.

PCR. PCR occurred in a solution containing the following: 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, 2 mM MgSO4, 5 mM MgCl2, 0.1% Triton X-100, 0.2 mM deoxynucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 0.5 μg/ml bovine serum albumin, 2.5 U of Taq DNA polymerase. The amount of template used was 1.0 μg per 150 μl of the reaction mixture. The reactions occurred in a Perkin-Elmer 9600 thermal cycler for 30 cycles. PCR was done with the set of HIV-1 IIB-derived primers SK68 (7801 to 7820, region gp41 Env) [5'-AGCAGCGAGGACGACATGCG-3'] and SK69 (7922 to 7942, region gp41 Env) [5'-CCACAGCTGAGAATTGCCAGG-3'].

RESULTS

Syncytium formation in the primary culture of ovarian carcinoma cells from ascites fluid. Ascites fluid samples obtained from ovarian carcinoma patients I to XXX were labeled ASC I to ASC XXX, respectively. Ascites fluid samples obtained from the mucinous cystadenoma patients were labeled MCI to MCV, and that obtained from the Brenner tumor patient was designated BREN I. The primary cell cultures of ASC cells contained large numbers of erythrocytes, lymphocytes, and epithelial and mesothelial cells and a very few cells of the cancer cell-like morphology (Fig. 1A). After 1, 2, and 3 days, cell culture medium was replaced with the fresh medium. Since erythrocytes do not attach to the plastic and cytotoxic lymphocytes attach poorly, the population of cells which remained attached to the plastic on day 4 of incubation contained mainly colonies of cancer cells, single fibroblasts, and some cells with the morphology of cytotoxic lymphocytes or macrophages. Groups of epithelial and mesothelial cells were also visible. On day 6, large colonies of quickly proliferating ascitic cancer cells were well visible (Fig. 1B). In the center of some of the colonies, small, poly nucleated syncytia were visible. At this stage, cells were trypsinized, resuspended in fresh medium, and transferred into the new culture flasks. During the next week, the cells were reaching confluence and morphologically resembled ovarian cancer cells. At the same time, some ASC cells enlarged while others formed syncytia containing two to eight nuclei (Fig. 1C). These fast-proliferating cells were merely contaminated with small, elongated normal epithelial or mesothelial cells, which proliferated poorly and died quickly after the first fusion and passed out of the cancer cells. After 3 weeks, all cancer cells became highly enlarged, and 50% of the cells formed syncytia. After 4 to 8 weeks, 90 to 95% of the ASC cells formed syncytia, which reached the size of 200 to 500 of the ovarian cancer cells and contained 3 to 20 nuclei (Fig. 1D and E). In 4 of 30 samples of ascites fluid tested, syncytia started to constitute as soon as after 2 to 4 days. It has been noted that all four patients exhibited a very large number of cancer cells in the ascites fluid, which was consistent with an advanced stage of the disease and the large volume of the malignant ascites fluid.

The process of syncytium formation was synchronized with strong vacuolization of the cytoplasm, formation of a dense, granular cytoplasmic ring around the nucleus, and formation of long, worm-like granular tubules “budding” from the cell (Fig. 1D and E). All tubules had the same perimeter (1 μm) and the worm-like appearance. A similar foamy-like structure of the syncytia was observed with 30 independently tested patients (ASC I to ASC XXX), which suggests that the phenomenon of cell fusion is common for the ovarian cancer cells isolated from ascites fluid. One of the ASC samples (ASC VI) formed syncytia as early as after 24 h of cell growth in vitro. Syncytia were formed in the cell culture for 2 months. During the last weeks of that period, some of the syncytia were reaching giant size, which was followed by apoptotic changes in the nucleus and lysis of the syncytia. Some cells did not form
syncytia, but were enlarged. Disintegration of the cytoplasm and apoptotic changes in these cells after 2 months of cell culture were also clearly visible (Fig. 1F).

MC cells did not form colonies and died after 2 to 3 days. Cells isolated from the patient with the Brenner tumor formed colonies after 3 to 5 days in cell culture, but did not form syncytia. Thus, syncytia were formed by ovarian cancer cells, but not by nonmalignant ovarian tumors. Additional control experiments were performed with ascites fluid obtained from patients with ovarian hyperstimulation and with ascites fluid obtained from the patient with atypical chronic lymphocytic leukemia. Ascites fluid produced by the patients with ovarian hyperstimulation contained large numbers of erythrocytes, lymphocytes, and fibroblasts. These normal cells proliferated...
poorly in the cell culture medium, did not form syncytia, and did not survive for longer than 7 days. Ascites fluid that originated from the leukemia patient contained several lymphoid blast cells and erythrocytes. None of the cells attached to the plastic or formed syncytia. Another group of control experiments included cell culture of blood cells. Blood cells obtained from the ASC donors did not form syncytia. These data exclude nonspecific fusion of cells due to the general viral contamination. Since cell cultures were not prepared from the ovarian cancer tissue, the ability of ovarian cancer cells to fuse and form syncytia has not been verified. The fact that the established cell line CaOV-3 formed some syncytia (as described below) might suggest that the ovarian cancer cells have the ability to form syncytia.

**Reactivity of the anti-HIV-1 gp120 MAb with ASC proteins.** Anti-HIV-1 MAb 5023 detected, by Western blotting, antigens p120, p42, and p25 in the protein extracts of all ASC syncytia (Table 1 and Fig. 2, lanes 1 to 4). The same proteins were detected in cancer tissue isolated from the patient OVI (donor of ASC VI) (Fig. 2, lane 9) and all other samples of ovarian cancer cells (Table 1). Antigens p120, p42, and p25 were associated selectively with ASC and with ovarian cancer cells. Ascites fluid, which was obtained directly from 26 patients and contained very few cancer cells, but a very large number of erythrocytes, lymphocytes, fibroblasts, and mesothelial cells, did not react with MAb 5023 (Table 1 and Fig. 2, lanes 5 to 8). Only 4 of 30 patients, all with a large number of cancer cells in the ascites fluid, tested RAK positive (Table 1). Thus, the antigens p120, p42, and p25 are expressed by ASC cells and are absent in normal cells. The last conclusion has been strongly supported by the lack of antigens RAK in normal epithelial cells obtained from the breast milk, normal skin, and normal ovary as well as other normal tissues (Table 1) (17, 20, 21, 24, 25). The results obtained with MAb RAK-BrI were identical to those obtained with MAb 5023 (not shown), which is consistent with the previously shown idiotypic similarity of both MAbs (17–21, 24, 25).

**Immunofluorescence staining of ASC syncytia with MAb RAK-BrI and the anti-HIV-1 gp120 MAb.** In indirect immunofluorescence staining, both MAb RAK-BrI (Fig. 3A and B) and the anti-HIV-1 gp120 MAb (MAb 5023) (Fig. 3C) very strongly labeled the cytoplasmic region adjacent to the nucleus.

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**TABLE 1.** Western blot detection of antigens RAK

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<th>Cell type</th>
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</tr>
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</tr>
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<td>Ascites fluid – cells</td>
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</tr>
<tr>
<td>ASC</td>
<td>30/30</td>
</tr>
<tr>
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<td>5 (+/-)/5</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>30/30</td>
</tr>
<tr>
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<td>0/3</td>
</tr>
<tr>
<td>Normal skin</td>
<td>0/6</td>
</tr>
<tr>
<td>Normal lymphocytes</td>
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<tr>
<td>Normal epithelial cells</td>
<td>0/15</td>
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<tr>
<td>Normal omentum</td>
<td>0/3</td>
</tr>
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</table>

*a* Ascites fluid directly collected from patients with ovarian cancer.

*b* Syncytia growing in the cell culture.

*c* Viral pellet obtained after ultracentrifugation of ASC culture medium (160,000 x g for 1 h).

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**FIG. 2.** Western blotting of antigens p120, p42, and p25 with the anti-HIV-1 gp120 MAb. Lanes: 1 to 3, ASC from different patients after 4 weeks marked by Roman numbers I, II, and V, respectively; 4, ASC VI after 2 weeks in cell culture; 5 to 8, ascites fluid directly obtained from the same patients; 9, solid ovarian cancer from the donor of ASC VI. *M*ₙ are shown to the left (thousands).
and much more weakly labeled the cytoplasm (Fig. 3). Cytoplasmic junctions formed during the fusion of ASC cells were clearly visible (Fig. 3A and B). Two types of syncytia were observed. In the first type, nuclei were localized in the center, and strong fluorescence staining with MAb RAK-BrI was restricted to the center of the cell (not shown). In the second type, nuclei were shifted to the one side, where the strong fluorescence was also observed (Fig. 3B and C). Intracellular deposits, strongly labeled with MAb RAK-BrI, were frequently visible in syncytia remaining in the cell culture for 6 to 8 weeks (Fig. 3C). The characteristic pattern of fluorescence was specific for MAb RAK-BrI, since the control MAb C63.3, which recognizes cellular and nuclear antigens (22), randomly stained both the cytoplasm and nucleus (not shown). The control MAb 5025, which does not recognize cancer antigens (19), did not bind to ASC cells.

The results obtained indicate that ASC syncytia synthesize high quantities of the MAb RAK-BrI and anti-HIV-1 gp120 MAb-recognized antigens. Strong labeling of the cytoplasmic bridges connecting two cells (Fig. 3A and B) suggests the role of these antigens in cell fusion. Perinuclear localization of fluorescence corresponded in general with localization of the viral particles (as described below).

**Effect of MAb RAK-BrI and anti-HIV-1 MAb 5023 on syncytium formation.** To determine whether the ASC antigens, which reacted in Western blotting, as well as in fluorescence
staining within epitope-specific and HIV-1-neutralizing MAbs, play any function in cell fusion, ASC cells (ASC III, ASC V, ASC VI, ASC XXV, and ASC XXX) grown for 2 weeks in cell culture were exposed to the anti-HIV-1 MAb (MAb 5023) or MAb RAK-BrI (10 or 20 μg/ml). After 3 to 5 days of cell incubation, changes in the behavior of cells exposed to any of the MAbs tested were clearly visible (Fig. 4). Cells exposed to MAb 5023 (Fig. 4A) or MAb RAK-BrI (not shown) were mainly mononuclear and formed a monolayer typical for cancer cells growing in vitro. No differences between 10 and 20 μg of the antibodies per ml were observed. Control cells, grown either in the absence of any MAb or in the presence of the control MAb 5025 or MAb C63.3 (Fig. 4B), formed typical syncytia. In addition to the effects described above, the nuclei of cells exposed to the anti-HIV-1 MAb contained well-dispersed chromatin, which is typical for healthy dividing cells (Fig. 4A). Nuclei localized within syncytia showed condensed, centrally localized chromatin not involved in mitosis (Fig. 4B). The experiments indicated that fusion of ASC cells was mediated by HIV-1-like antigens RAK.

PCR with HIV-1-derived primers. The reactivity of ASC antigens with the anti-HIV-1 gp120 MAb and with MAb RAK-BrI, as well as strong fluorescence staining of the syncytia with both MAbs, strongly suggested that ASC cells may contain DNA sequences homologous to HIV-1. Therefore, PCR analysis was performed with HIV-1 Env-derived primers (Fig. 5). HIV-1 gp41-derived primers initiated PCR in the presence of DNA isolated from ASC cells of different patients and from ovarian cancers of the ovary (Fig. 5, lane 3). The amplified DNA fragments were of similar size (142 bp) to those in HIV-1-infected DNA (Fig. 5, lane 5). PCR was negative with DNA isolated from normal ovary cells (Fig. 5, lane 4) or any other normal cells (17, 24) (Table 2).

In 9 of 30 patients, DNA was extracted from the fresh ascites
fluid as well as ASC cells. PCR amplification was obtained with all DNA samples obtained from the ASC cells, but in only 7 of 9 samples obtained from the fresh ascites fluid (Table 2). The four highly positive samples were obtained from patients with advanced disease, the ascites fluid of which contained a large number of cancer cells. The next three PCR-positive DNA samples originated from patients with a low density of cancer cells.

PCR fragments amplified in ovarian cancer DNA and in ascites cell DNA showed over 90% homology to the conserved region of the HIV-1 gene for the transmembrane protein gp41 (Fig. 6A). It is noteworthy that identical sequences were amplified in both ovarian cancer DNA and in ASC DNA from the same patients, but polymorphism of the fragments amplified in various patients was clearly visible (Fig. 6A [two patients’ data are shown as an example]). Translation of the HIV-1-like DNA sequences into the amino acid sequences revealed peptides with conserved isoleucine in position 11 replacing methionine present in HIV-1 and aspartic acid in position 36 replacing glutamic acid (Fig. 6B).

**TABLE 2. PCR Amplification of HIV-1-like DNA sequences**

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<th>DNA origin</th>
<th>No. positive/no. tested by PCR</th>
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<td>ASC</td>
<td>18/18</td>
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<tr>
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<td>30/30</td>
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<td>Normal skin</td>
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<tr>
<td>Normal lymphocytes</td>
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<td>Normal epithelial cell</td>
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<tr>
<td>Normal omentum</td>
<td>0/2</td>
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</table>

*Ascites fluid directly collected from patients with ovarian cancer.

**FIG. 5.** PCR amplification of ovarian cancer cell DNA with primers SK68 and SK69 derived from HIV-1 gp120. Lanes: 1 to 3, DNA isolated from ASC cells; 4, DNA from ovarian cancer; 5, DNA from normal ovary; 6, DNA from HIV-1-infected cells; 7, ladder marker (125 bp).

**FIG. 6.** Sequences, amplified with HIV-1 gp41-derived primers SK68 and SK69 in ovarian cancer DNA and ASC DNA from two different patients. (A) The sequences identical to that of HIV-1 H1b are marked by stars. The positions of primers are marked by dashed lines. (B) Amino acid sequence encoded by the HIV-1-like sequences.

**Electron microscopic detection of virus-like particles.** Fusion of cells isolated from ascites and formation of the highly vacuolated, giant syncytia strongly suggested that an unknown virus might be activated in ascitic cells growing in vitro. To analyze viral particles, cell culture media from ASC I to VI and from ovarian carcinoma cell line CaOV-3 (control) were collected and ultracentrifuged. The pellets were negatively stained, as described in Materials and Methods, and tested by electron microscopy (Fig. 7).
Spherical, membrane-covered particles, 100 to 110 nm in diameter, were detected in the ASC growth medium (Fig. 7A to C). These virus-like particles showed prominent exterior membranes, some with protein projections. To eliminate the possibility that the isolated particles originated from the ascitic fluid of the patients, instead of being of ASC (cellular) origin, an established ovarian cancer cell line, CaOV-3, was used as a control. Similar, spherical particles, slightly larger in size (120 nm) were detected in cell growth medium (Fig. 7D and E), which suggests that ovarian cancer cells growing in vitro produce virus-like particles similar to those identified in ASC culture medium.

To further confirm the cellular origin of the particles found in ASC culture medium, thin-section analysis of ASC syncytia was performed. Electron microscopy revealed a large number of spherical viral particles, but smaller in size (80 to 90 nm), in the cytoplasm adjacent to the nucleus (Fig. 8). These viral particles showed a hexagonal structure, with a dense central ring-shaped core surrounded by an inner translucent capsule and outer electron-dense shell (Fig. 8). The outer shell consisted of regular granules with longer, symmetrically distributed spindles (Fig. 8B). No budding particles were found on the cell surface. In all syncytia, viral particles were detected mainly in the cytoplasmic harbors and were surrounded by the irregularly shaped nucleus of adjacent nuclei (Fig. 9A and B). Viral particles budding from the plasma membrane located between two nuclei (Fig. 9B), as well as from plasma membrane vesicles distributed in various regions of the cytoplasm, were also observed (Fig. 9). More regular shapes of the viral particles budding from intracytoplasmic membranes were comparable with those of the particles found in the cell culture medium (Fig. 7). Several vacuoles with virions budding into cellular vacuoles were also visible (Fig. 9C).

**Morphological transformation of normal breast cells by ASC-produced factor.** Cell culture media collected from ASC III, ASC V, and ASC VI were centrifuged at 6,000 × g for 1 h in order to eliminate cell debris. Normal breast cells (cell line Hs578Bst) were trypsinized and transferred into ASC III, ASC V, and ASC VI cell growth media. One control group of normal breast cells was transferred into the cancer cell culture medium, and the other group was transferred into the normal cell culture medium. The following observations were made. (i) Normal breast cells growing in cancer cell medium proliferated poorly and died after 14 days. (ii) Normal breast cells growing in normal cell culture medium (enriched with growth factors) proliferated into the monolayer after 10 days (Fig. 10A). (iii) Normal breast cells transferred into the ASC cell growth medium underwent significant changes in morphology and behavior. These cells significantly enlarged and underwent several karyokinetic divisions without cytokinesis. Instead, the cells formed long cytoplasmic extensions that participated in cell fusion. During the fusion, nuclei were “injected” from one cell to another (Fig. 10B). The syncytia which were formed early by the transformed breast cells (2 to 21 days of exposure to ASC medium) were irregular in shape and contained several cytoplasmic extensions (Fig. 10B). Syncytia remaining in the cell culture for 1 to 2 months became more regular and did not contain cytoplasmic extensions. The size difference between normal breast cells and the transformed cells (syncytia) was visible after indirect fluorescence staining with MAb RAK-BrI (Fig. 10C and D). In this transformed form, the breast cells survived in the cancer medium for 2 to 3 months. Syncytia formed with ASC III, ASC V, or ASC VI looked similar. No transformation of normal cells was obtained in the cell growth medium collected from breast cancer cell lines MCF7 and SkBr3 or from ovarian cancer cell lines. These results suggest that viral particles identified in ASC medium may be responsible for the transformation of breast cells.
DISCUSSION

The presence of various viral particles (1, 5, 7–9, 12, 13) and viral sequences (6) in human cancers was reported by several laboratories. The function, if any, of these viruses in the process of malignancy was not established. None of the reports demonstrated the presence of any viral particles in ovarian cancer. Data presented in this publication strongly imply that a yet uncharacterized, latent virus might be present in ovarian cancer cells. Large and highly vacuolated syncytia were formed only by ovarian cancer cells, but not by nonmalignant Brenner tumor or mucinous cystadenoma cells. Since all ASC, BREN, and MC cells originated from the intraperitoneal ascites, the role of cells other than cancer cells in formation of syncytia is unlikely. The fact that normal cells present in ascites fluid obtained from the patients with ovarian hyperstimulation did not form syncytia further confirms the conclusion that only ovarian cancer cells isolated from ascites fluid formed syncytia. In other control experiments, no syncytia were observed in ascites fluid obtained from patients with chronic lymphocytic leukemia. It is noteworthy that syncytia were never found in cervical SiHa; breast MC7 or SKBr3; lung 1810; colon SW 707, SW 1116, and SW 948 carcinoma; or melanoma HS 294, A 875, and WM 266-4 cell lines grown in the cell culture for years. As we reported here, less than 0.5% of ovarian cancer cells (CaOV-3) remaining in the cell culture for a long time also form syncytia and produce viral particles similar to those found in ASC cells.

The syncytia that were formed by cancer cells isolated from ascites fluid of ovarian cancer patients were morphologically very similar to the syncytia induced by spumavirus (foamy virus)-infected cells (10, 14, 28). Spumaviruses are phylogenetically related to lentiviruses (HIV) and oncoviruses, all of which are able to induce syncytia (reviewed in reference 14). Virus-mediated syncytium formation is the poorly understood chain of events leading to cell fusion. In the case of HIV-1, syncytium is formed when the gp120 antigen expressed on the cell surface of the infected T lymphocyte is recognized by the CD4 receptor of another T lymphocyte. Syncytium formation by HIV-1-infected T lymphocytes has been frequently observed in vitro, but is not seen in vivo (26).

It is noteworthy that ovarian cancer cells express antigens p120, p45, and p25, which exhibit immunologic, biochemical, and genetic homology to HIV-1 antigens (19, 20). None of these HIV-like antigens are present in normal tissues (17, 24, 25). Although RAK antigens exhibit homology to HIV-1 antigens, these cancer markers can be easily distinguished from HIV-1 infection by using any antibody directed against the glycosylated form of HIV-1 gp120 or MAb 5025 (19) or any other anti-HIV-1 MAb which does not cross-react with cancer antigens. Moreover, MAb RAK-BrI binds to RAK p120, p42,
and p25 in cancer cells, but only to gp160 or gp120 of HIV-1 (18), which automatically eliminates a possibility of infection. In the current studies, HIV-like antigens were detected in all ASC syncytia. Since HIV-1-like antigens are absent in normal cells, RAK positivity of ASC syncytia should suggest that these cells represent, in fact, cancer cells. HIV-like antigens were undetectable in ascites fluid which was directly obtained from the patients with a small number of cancer cells in the ascites fluid. The lack of p120, p42, and p25 in cells other than cancer cells is consistent with previous observations (17, 24, 25). Subsequently, patients with a high density of cancer cells in the ascites fluid tested RAK positive.

Immunologic cross-reactivity of the anti-HIV-1 gp120 MAb with cancer antigens and of MAb RAK-BrI (directed against breast cancer antigens) with HIV-1 is due to the presence of the epitope GRAF, common for cancer and HIV-1 envelope protein gp120 (18). Strong immunofluorescence labeling of the perinuclear cytoplasm of ASC syncytia with both MAb RAK-BrI and the anti-HIV-1 gp120 MAb strongly suggests that the antigens with epitopic homology to HIV-1 might be associated with virions synthesized in the cytoplasm adjacent to the nucleus. Moreover, strong labeling of cellular junctions participating in the cell fusion might imply there is a role of HIV-like cancer antigen p120 in fusion of ASC cells. The fact that the HIV-1-neutralizing anti-gp120 MAb as well as MAb RAK-BrI
inhibited syncytium formation by ASC cells strongly suggests that the ASC p120 may play a role in cell fusion similar to the role gp120 plays in HIV-1-infected cells.

DNA sequences with 90 to 96% homology to HIV-1 Env were recently detected by PCR in breast cancer and in prostate cancer cells (17, 24) and deposited in the GenBank as gene(s) RAK alpha. Current studies indicated that HIV-1-like sequences, amplified with HIV-1 gp41-derived primers are also present in DNA isolated from the solid cancers of ovary, as well as from ovarian cancer cells isolated from ascites fluid. The fragment of DNA amplified with primers SK68 and SK69 is relatively conserved in various isolates of HIV-1; therefore, no homology with the specific HIV-1 strain can be established. DNA obtained from normal ovaries tested PCR negative, similar to DNA isolated from normal cervix, vulva, vagina, uterus, prostate, skin, and lymphocytes and other normal tissues (17, 24).

DNA sequences identified in ASC DNA and ovarian cancer DNA showed over 90% homology to HIV-1 gp41. Note that similar point mutations were conserved in different ovarian cancer patients. Codons 11, 26, 28, and 36 were also mutated in prostate cancer (17) and breast cancer (18) DNA. We have shown before that PCR markers RAK are expressed by 95% of breast, gynecological (24, 25), and prostate (17) cancer patients, which automatically excluded a possibility of HIV-1 infection. Infection with any known virus or contamination of ASC cultures with bacteria or mycoplasma had been eliminated by electron microscopy done by Advanced Biotechnology, Inc.

The peptide encoded by the HIV-1-like sequences contains an isoleucine at position 11 replacing methionine and aspartic acid at position 36 replacing glutamic acid. Note that translation of the prostate and breast cancer sequences (17, 18) revealed the same amino acids at positions 11 and 36, which strongly suggests the cancer virus is highly homologous but distinct from HIV-1.

PCR primers derived from the various parts of spumaviruses (regions Bel 1 and Bel 2 and the long terminal repeat) did not amplify ASC or ovarian cancer DNA, which eliminates infection with a known foamy virus and implies that the identified virus is not genetically related to foamy virus. However, formation of the syncytia relatively late in the culture of ASC cells and strong vacuolization of syncytia more closely resemble the cytopathic effects of spumavirus infection than the effects of an infection by lentiviruses or oncoviruses.

Spumaviruses were never found in the fresh cancer tissue and are known to replicate only in vitro (reviewed in reference 14). The viral particles described were detected only in ASC cells grown for 2 to 5 weeks in vitro. Viral particles were synthesized in the perinuclear cytoplasm, preferably in the nuclear invaginations of the cytoplasm. Several viral particles were found in the plasma membrane adjacent to two nuclei, which suggests that these viral particles may participate in cell fusion.

The presence of the dense central core and of the inner highly visible translucent capsid, wrapped with the viral envelope, matches the general structure of the spumaviruses (28). However, the projections of the envelope were different than

FIG. 10. Morphological transformation of normal breast cells by ASC cell growth medium. Results are shown for cells grown in the absence (A and C) or in the presence (B and D) of ASC medium. (A and B) Phase-contrast microscopy. Magnification, ×816. (C and D) Indirect fluorescence staining with anti-HIV-1 MAb. Magnification, ×2,220.
those found on classical foamy virus (28). The hexagonal geometry of the viral particles may suggest the icosahedral structure of the virus. The presence of the external membrane was visible in the particles isolated from the ASC culture medium. No particles were found to be budding directly from the cell surface. Several vacuoles containing viral particles were morphologically similar to those described by Gajdusek and collaborators (10) in foamy virus-infected chimpanzees. Whether the worm-like structures budding from the syncytia exhibit any physiological function remains to be established.

Very few viral particles were also detected in the cell culture medium of CaOV-3 cells, which sporadically form syncytia in the cell culture. Since no more than 0.5% of CaOV-3 cells form syncytia (and probably activate the virus), we were not able to detect the viral particles inside the CaOV-3 cells. The spherical particles of the virus-like shape identified in cell cultures of ASC cells and ovarian CaOV-3 cancer cells suggest that this unknown virus might be present in the latent form and might play a role in the etiology of ovarian cancer. Genetically, this new virus seems to be related to HIV-1, but functionally it induces foamy syncytia, similar to those formed by spumaviruses. Although spumaviruses were never documented to be involved in pathogenic processes, they were predominately isolated from sick individuals. Since spumaviruses replicate poorly in vitro, knowledge about these viruses remains far behind the current knowledge about other members of the Retroviridae family. The fact that progressive encephalopathy and myopathy were observed in transgenic mice expressing a gene of human foamy virus (4) strongly suggests that spumaviruses may play a role in pathogenesis. A simple experiment described in this report indicated that culture medium collected from syncytium-forming ovarian cancer cells (ASC) transformed morphologically and functionally normal breast cells, which also started to form syncytia. Further exploration of the molecular structure and biology of the viruses detected in ovarian carcinoma cells might bring new progress in understanding the etiology and improving the diagnoses of ovarian malignancies.

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