

The Insulin-like Growth Factor 1 Receptor Induces Transformation and Tumorigenicity of Ovarian Mesothelial Cells and Down-Regulates Their Fas-Receptor Expression

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ABSTRACT

Cell proliferation and papillogenesis are growth factor-sensitive events in the ovarian mesothelium, the tissue source of ovarian epithelial cancer. To further investigate the regulation of cell proliferation in this tissue, rabbit ovarian mesothelial cells (OMC) were transfected *in vitro* with a CVN expression vector carrying the human gene for insulin-like growth factor 1 receptor (IGF-1R). The growth characteristics of IGF-1R transfectants (OMIR) and their response to IGF-1 were then compared with those of OMC in serumless HL-1 cultures. OMIR cells formed epithelial-like colonies and, even when nonconfluent, produced tridimensional structures reminiscent of papillae seen in ovarian serous epithelial tumors. After 3 and 7 days of exposure to IGF-1, OMIR cells grew ~20-fold ($P < 0.05$), and papillogenesis was 15- to 25-fold over similar events in OMC, respectively. Exposure to treatment with antisense oligonucleotides against IGF-1R mRNA inhibited OMIR growth rate by 70%. Western immunoblotting and flow cytometry revealed higher expression of IGF-1R in OMIR cells than in OMC. The reverse was true when Fas-receptor expression was evaluated. OMIR cells were clonogenic in 15% serum-rich soft agar assay (OMIR:OMC colony-forming ratio 150–200:1), and tumorigenic in nude mice in which high-grade carcinomas with occasional lung metastases were observed. These data suggest that IGF-1R plays a role in ovarian epithelial carcinogenesis. The overexpression of this receptor induces transformation and morphogenesis of OMCs via an autocrine mechanism. IGF-1R may down-regulate the Fas expression rendering transformed ovarian mesothelial cells resistant to apoptosis.

INTRODUCTION

The ovarian surface epithelium is a modified OM² believed to be the source of ovarian epithelial cancer (1, 2). This tumor is highly aggressive and usually presents late in its development (3). Patients with this form of cancer have a short median survival time after diagnosis, and even when the tumor is clinically localized, the 5-year relative survival rate is less than 50%, regardless of therapy (4). The American Cancer Society estimates that there were 26,800 new cases of ovarian cancer in 1998 in the United States, with 14,200 estimated cancer deaths in the same year (5). These observations attest our current limited understanding of ovarian cancer pathobiology.

Sequential activation of cell receptors, including the IGF-1R, oncogenes, and tumor suppressor genes have been implicated in ovarian epithelial carcinogenesis (6–9). The expression of IGF-1 and of IGF-1R binding proteins have been reported in three different ovarian carcinoma cell lines (OVCAR-3, OVCAR-7, and PEO4; Ref. 10). It has been shown that CAOV-3 and OVCAR-3 cells produce endoge-

nous IGF-1 and grow autonomously in serumless media. Their growth in these conditions, however, is further stimulated by the addition of IGF-1 (11). The treatment with IGF-1R mRNA antisense oligodeoxynucleotides markedly inhibits the proliferation of these cells both in serumless media and in the presence of IGF-1. Such inhibition corresponds to a reduction in the amount of detectable phosphorylated IGF-1R (11). These data together suggest that the IGF-1/IGF-1R system, may have a prominent role in the proliferation and transformation of normal rabbit ovarian mesothelial cells. It has also been postulated that another function of the IGF-1R is to protect cells from undergoing apoptosis (12, 13). In this investigation, we report the transformation of normal rabbit ovarian mesothelial cells after their transfection with a plasmid constitutively expressing the human IGF-1R (OMIR cells). These cells were able to grow in soft agar and to produce large debilitating tumors when injected s.c. in nude mice. In addition, the overexpressed IGF-1R was associated in these cells with a decreased expression of the Fas receptor and consequent inhibition of apoptosis.

MATERIALS AND METHODS

Cell Isolation and Culture. Four rabbit ovaries were used to obtain OMCs. The cells were isolated from ovaries of 4–5-month-old New Zealand White female estrous rabbits, as described previously (14). Cells were grown in CM199, containing 15% fetal bovine serum, at 37°C. After culturing, the phenotype of OMCs and OMIR cells was evaluated immunohistochemically using anticytokeratin and antivimentin antibodies, and electron microscopy, as described previously (9, 15). In brief, OMCs and OMIR cells were fixed *in situ* at 4°C in 2.5% glutaraldehyde. After washing in 0.1 M phosphate buffer, fixation in 1% osmium tetroxide for 1 h at 4°C, and dehydration, the tissue was embedded in LX112 epoxy resin (Ladd Corporation, Burlington, Vt). Thin sections were cut and stained for 10 min in 8% aqueous uranyl acetate and for 5 min in Reynold's lead citrate. Sections were examined with a Philips CM10 transmission electron microscope.

Plasmid Transfection. Rabbit OMCs were transfected, using the suspension method, with a CVN expression vector containing the full-length coding sequence of the human IGF-1R-cDNA and the neomycin resistance gene, both under the control of the SV40 early promoter. This plasmid was kindly provided by Dr. Renato Baserga (Jefferson Cancer Center, Philadelphia, PA). After 48 h, 0.6 mg/ml G418 was added to the cells to obtain stable transfectants.

Cell Growth and Papillogenesis Experiments. OMCs and OMIR cells (clones 1, 2, and 3) were seeded in medium containing 15% fetal bovine serum, at an initial density of $3-6 \times 10^3$ cells/cm². The cells were allowed to attach for 24 h and then arrested in HL-1 SFM. After the addition of 10 ng/ml IGF-1 (Life Technologies, Inc., Gaithersburg, MD), duplicate cultures were counted in a hemocytometer at 24, 48, and 72 h. In a successive experiment, OMC and OMIR clones 2, 3, and 8 were tested for growth in SFM without the addition of IGF-1. OMIR cells, maintained in SFM, were then exposed to sense (5'-AAG TCT GGC TCC GGA GGA) or antisense (5'-TCC TCC GGA GCC AGA CTT) IGF-1R mRNA oligodeoxynucleotides (40 ng/ml) for 48 h with and without the addition of IGF-1. In this experiment, the cells were counted using MTT (Sigma Chemical Co., Milwaukee, WI). This assay is based on reduction of MTT to formazan by enzymes present only in viable, metabolically active cells. After exposure to sense and antisense IGF-1R mRNA oligonucleotides, the medium was removed, and 0.1 ml of MTT (50 μg) was added to each well. After

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² The abbreviations used are: OM, ovarian mesothelium; OMC, OM cell; OMIR, transfected OMCs expressing human IGF-1R; CM199, complete medium 199; IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, TdT-mediated dUTP nick end labeling.

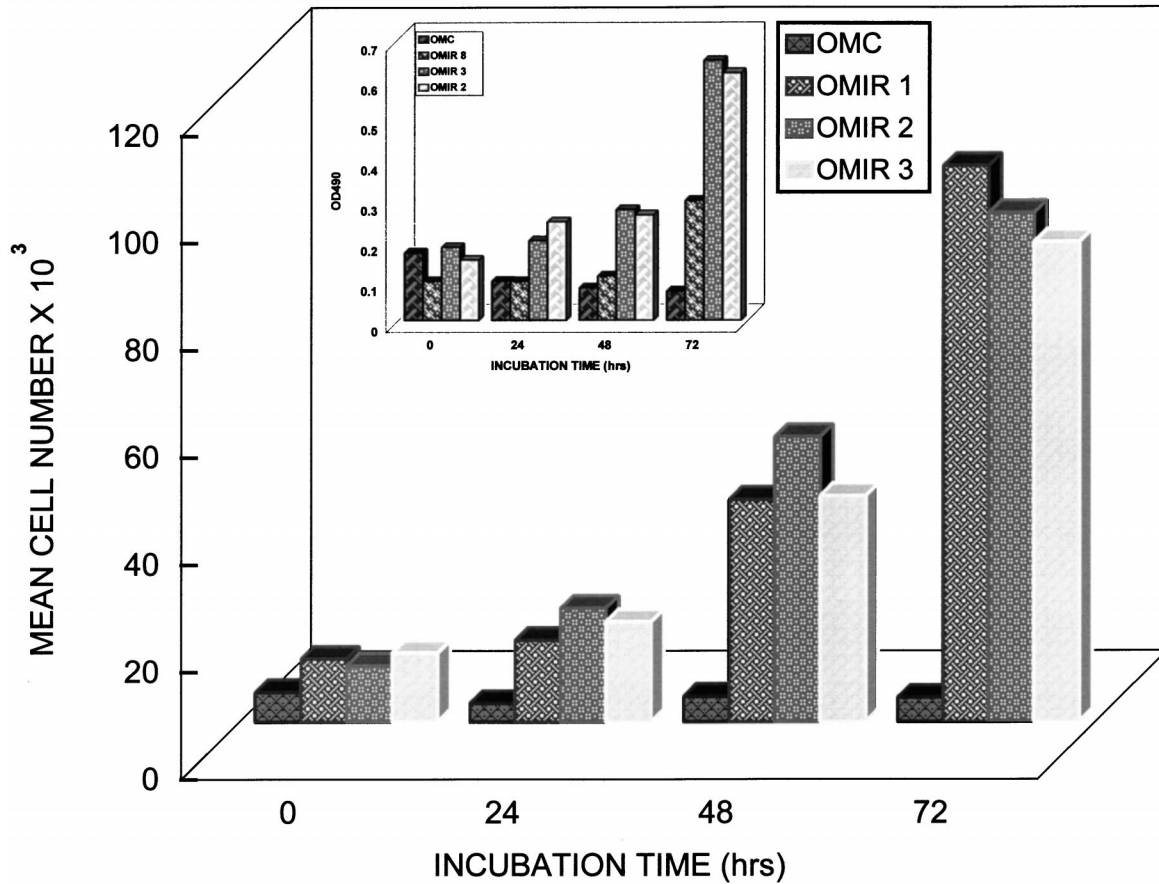


Fig. 1. Proliferation of OMC and OMIR clones 1, 2, and 3 in SFM with and without (*insert*) the addition of IGF-1 (20 ng/ml). The proliferation of OMIR clones is approximately 20-fold over that of OMC ($P < 0.05$).

incubation for 2 h at 37°C, the multiwell plate was centrifuged to pellet formazan crystal, and the medium was discarded. Then, 0.1 ml of DMSO was added to each well to dissolve the MTT formazan crystals. The absorbance of formazan at 540 nm was measured by Emax microplate reader (Molecular Devices, Menlo Park, CA). A mean of 18 replicates cultured from three experiments were used for statistical analysis. For

morphogenetic studies, OMC and OMIR clones were cultured in duplicates in CM199–15% fetal bovine serum for 7 days. Papillary-like processes, defined as the aggregation of cells into tridimensional processes arising from cell monolayers, were counted and reported as the number of processes/well at days 3, 4, 5, 6, and 7. Formation of papillary-like processes by OMC (passage 4) and OMIR clone 8 (passage 16) was also assessed in

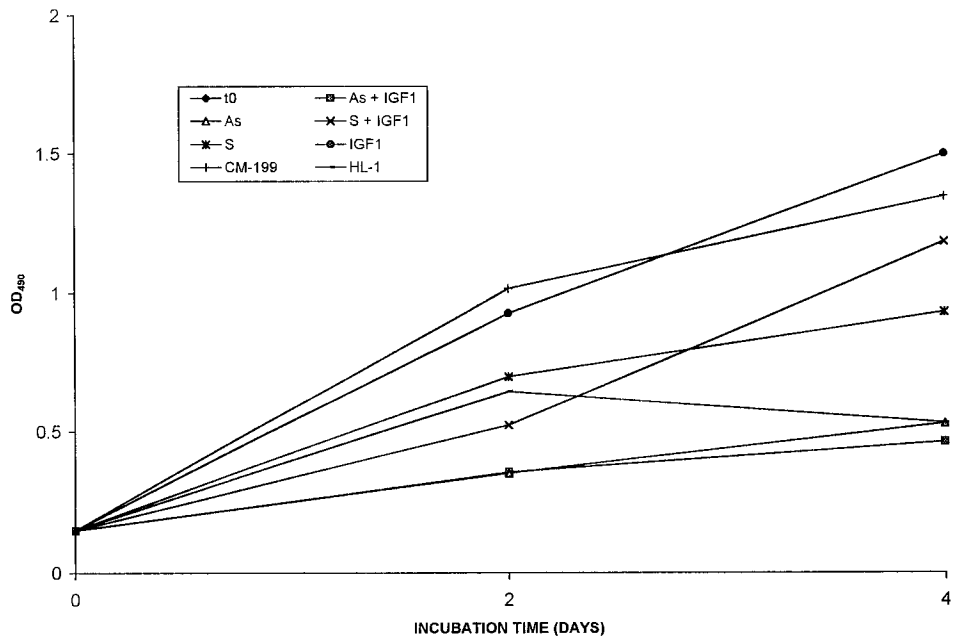


Fig. 2. Effect of sense (S) and antisense (As) IGF-1R mRNA oligonucleotides on the growth of OMIR cells. OMIR cells were maintained in SFM as described in the "Material and Methods" section. Where indicated, oligonucleotides (40 ng/ml) were added to the medium for 24 h before the addition of IGF-1 (10 ng/ml) at time zero. Medium containing oligonucleotides or IGF-1 was replaced daily. Cells were then counted at the indicated times. The data represent the mean of triplicate determinations from one of three representative experiments.

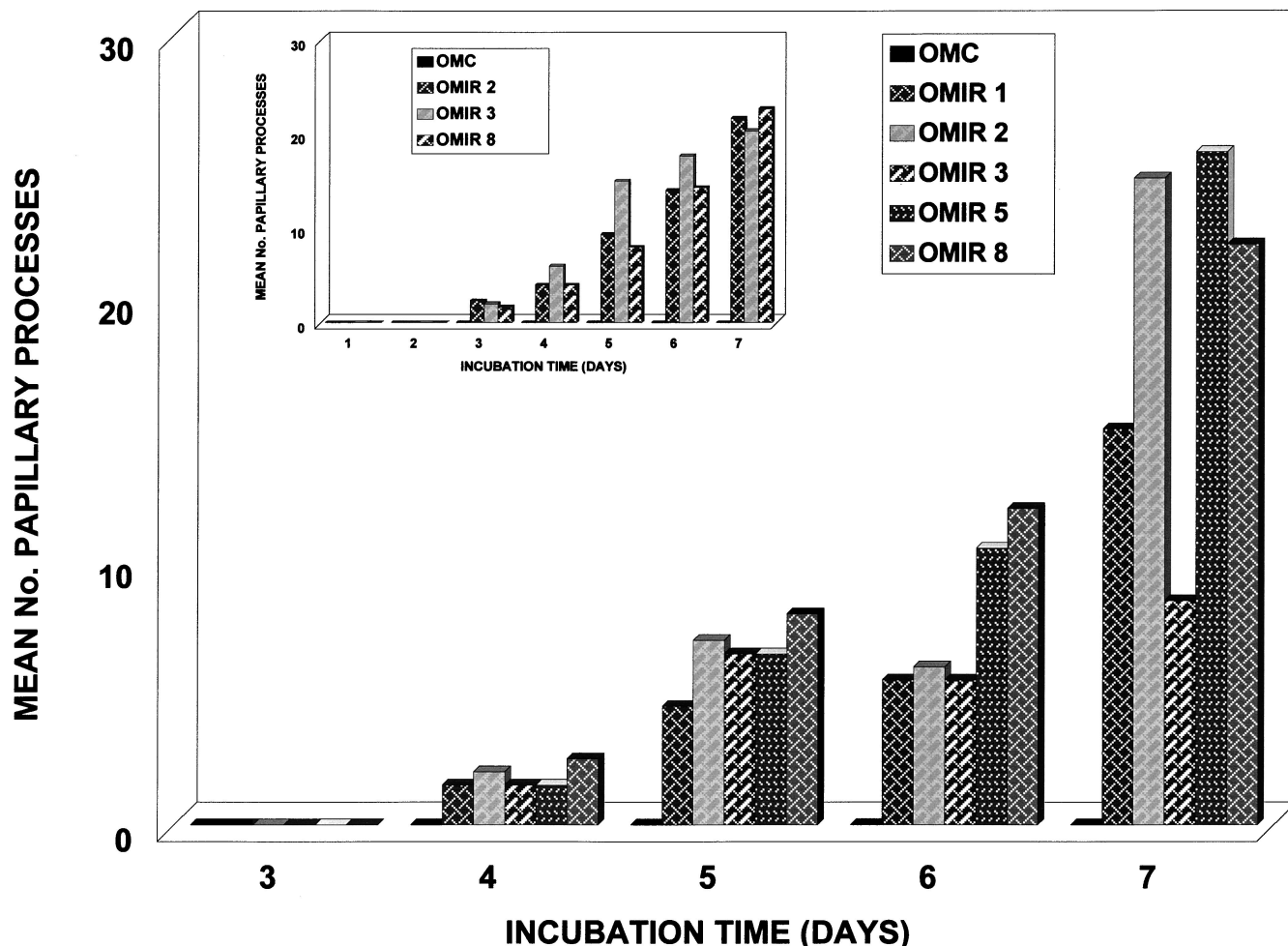


Fig. 3. Number of papillary processes produced by OMCs and OMIR cells cultured in 15% serum or in SFM with the addition of fibronectin (*insert*) for 7 days. In both experiments, papillogenesis of OMIR is approximately 15- to 25-fold over that of OMC ($P < 0.05$).

SFM in the presence of fibronectin (4 $\mu\text{g/ml}$). Papillary morphology was evaluated by transmission and scanning electron microscopy as described previously (15).

Measurement of IGF-1R and Fas-R. The expression of IGF-1R and Fas-R in OMCs and OMIR cells was analyzed by Western immunoblotting under reducing conditions as follows:

(a) cell lysates were obtained from the above cells exponentially growing in 10% serum;

(b) after clarification by centrifugation and protein concentration determination, 20 μg of protein were resolved on 4–15% polyacrylamide gradient gel by SDS-PAGE and electroblotted into a nitrocellulose filter;

(c) the filter was immunoblotted with mouse antihuman monoclonal antibodies to identify the IGF-1R α (Life Technologies, Inc.) and the M_r 40,000–50,000 cell membrane Fas receptor (Apo-1/Fas, DAKO Corp.), followed by antimouse horseradish peroxidase-conjugated antibody (Oncogene, Science, Inc. Mineola, NY); and

(d) antigen bound to nitrocellulose membrane polyvinylidene difluoride (PVDF) was detected using Vectastain (ABC immunoperoxidase system), and protein bands were visualized with a diaminobenzidine tetrahydrochloride DAB substrate.

Determination of IGF-1R and Fas-R expression on OMCs and OMIR cells was also obtained by flow cytometry. Dilutions and washings were carried out in RPMI 1640 containing 2% heat-inactivated FCS, 0.1% sodium azide, and 10 mM HEPES. Approximately 10^6 cells/sample, suspended in 50 μl of medium, were incubated at 4°C with an equal volume of IGF-1 and Fas monoclonal antibody. After 45', cells were washed twice in 500 μl of cold medium and 2 μg of F(ab')₂ goat antimouse IgG, and IgM FITC conjugate (Jackson ImmunoResearch, West Grove, PA) was added for 45' at 4°C.

Cells were washed again twice and resuspended in 300 μl of medium containing 1 $\mu\text{g/ml}$ propidium iodide (Sigma). From each sample, the green fluorescence of 10^4 cells was analyzed. Dead cells were removed from analysis by selectively gating on propidium-iodine fluorescence, according to forward and side-scatter parameters. Flow cytometry analysis was performed on a FACScan (Becton Dickinson) flow cytometer equipped with a 15 mW argon laser capable of three-color analysis. A FACStar^{plus} (Becton Dickinson) was used for cell sorting. Additional data analysis was done with a Cell Quest software package (Becton Dickinson).

Detection of Apoptosis by *in Situ* Hybridization Using TUNEL Reaction. Apoptosis of OMC and OMIR was evaluated by the *in situ* immunohistochemical detection and quantification at a single-cell level based on labeling of DNA strands breaks, after exposure to CH-11, a ligand for the Fas receptor. This was determined by TUNEL, using an *in situ* cell-death detection kit (Boehringer Mannheim, Indianapolis, IN). OMC and OMIR (clones 3 and 8) were trypsinized and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde [4% in PBS (pH 7.4)], rinsed with PBS, and incubated in permeabilization solution. After cross-reaction with TUNEL reaction mixture for 60 min at 37°C and cross-reaction with converter-alkaline phosphatase solution for 30 min at 37°C in a humidified chamber, the slides were reacted with alkaline phosphatase substrate solution for 5–10 min (Vector Laboratories, Burlington, MA), rinsed, and mounted under coverslip for analysis under light microscope.

Clonogenicity in Soft Agar. OMCs and OMIR cells were seeded at a density of $6 \times 10^3/35\text{-mm}$ plate in 15% fetal bovine serum on a top layer of 0.3% agar and a bottom support layer of 1% agar. The plates were incubated at 37°C. Colonies of greater than 10 cells were counted 7 and 14 days later.

Tumorigenicity in Nude Mice. To test the ability of OMCs and OMIR cells (clones 2, 3, and 8) to produce tumors *in vivo*, 7-week-old BALB/c nude mice were given injections s.c. with 5×10^6 cells. Twenty-four h before the injection, cells were incubated in SFM at 37°C. After trypsinization, cells were suspended in 0.2 ml of PBS and injected above the hind leg of the nude mice.

Statistical Analysis. The results obtained with the different OMIR clones were pooled for statistical evaluation and compared with the respective OMC controls by the ANOVA test. Differences were considered significant when $P < 0.05$.

RESULTS

Growth Characteristics of OMCs and OMIR Cells. Both OMCs and OMIRs were immunohistochemically positive for low-molecular-weight cytokeratin and vimentin supporting their mesothelial lineage. The growth characteristics of OMCs as compared with OMIR cells are shown in Fig. 1. When cultured in SFM, OMCs did not respond to the addition of exogenous IGF-1. Conversely, the growth response of OMIR transfectants to IGF-1 was ~20-fold greater ($P < 0.05$). Even in SFM alone, OMIR cells proliferated, although at a lower rate (~8-fold; Fig. 1, *insert*). The addition of antisense oligonucleotides against the IGF-1R mRNA decreased OMIR cells growth rate by 70%, both in the presence and absence of exogenous IGF-1 (Fig. 2). OMIR cells grown in 15% serum, even when nonconfluent, produced tridimensional papillary-like structures, whereas OMCs under the same conditions produced only a very few papillae (15- to 25-fold less; Fig. 3). When papillogenesis was assessed in SFM with the addition of fibronectin, OMCs failed to produce similar structures, whereas OMIR's papillogenesis rate remained unchanged (Fig. 3, *insert*).

Morphogenetic Studies. Scanning electron microscopy performed on 5-day-old OMIR cultures in SFM and fibronectin showed the emergence of microvilli-lined, and occasionally ciliated, papillary-like processes from adjacent monolayer cells (Fig. 4, A and B). Transmission electron microscopy of these tridimensional aggregates showed rounded cells, occasionally exhibiting pleomorphic nuclei and prominent nucleoli. The mesothelial nature of the cells was confirmed by the demonstration of desmosomes, intermediate filaments, occasional intracellular lumina, and microvilli (Fig. 4C).

Evaluation of IGF-1R and Fas-R Expression on OMCs and OMIR Cells. When examined by Western immunoblotting, OMIR clones revealed a distinct band of M_r 135,000 representative of the α subunit of the IGF-1R. Conversely, the OMCs revealed only a weak, indistinct band of the same molecular weight (Fig. 5A). Flow cytometry showed the OMIR cells to express more IGF-1R than OMCs (1:10 ratio; Fig. 5C). The opposite was observed for Fas-R, which was strongly expressed on OMCs but absent on OMIR cells by both Western and flow cytometry evaluation (Fig. 5, B and D).

Detection of Apoptosis by *in Situ* Hybridization Using TUNEL Reaction. OMCs underwent apoptosis after exposure to CH-11, a ligand for the Fas receptor, as demonstrated by TUNEL alkaline phosphatase assay (Fig. 6A). OMIR clones showed instead only rare apoptotic cells after exposure to the same ligand (Fig. 6B).

Clonogenic Assay. The cloning of OMIR cells in 15% serum-rich soft agar produced numerous colonies (Table 1), in a ratio of 150–200:1 as compared with OMCs.

Table 1 Number of colonies formed in soft agar

OMC		OMIR tumors studied		
Incubation time 37°C	n	Clone 1	Clone 2	Clone 3
1 wk	12	701	501	760
2 wk	20	2108	1508	1442

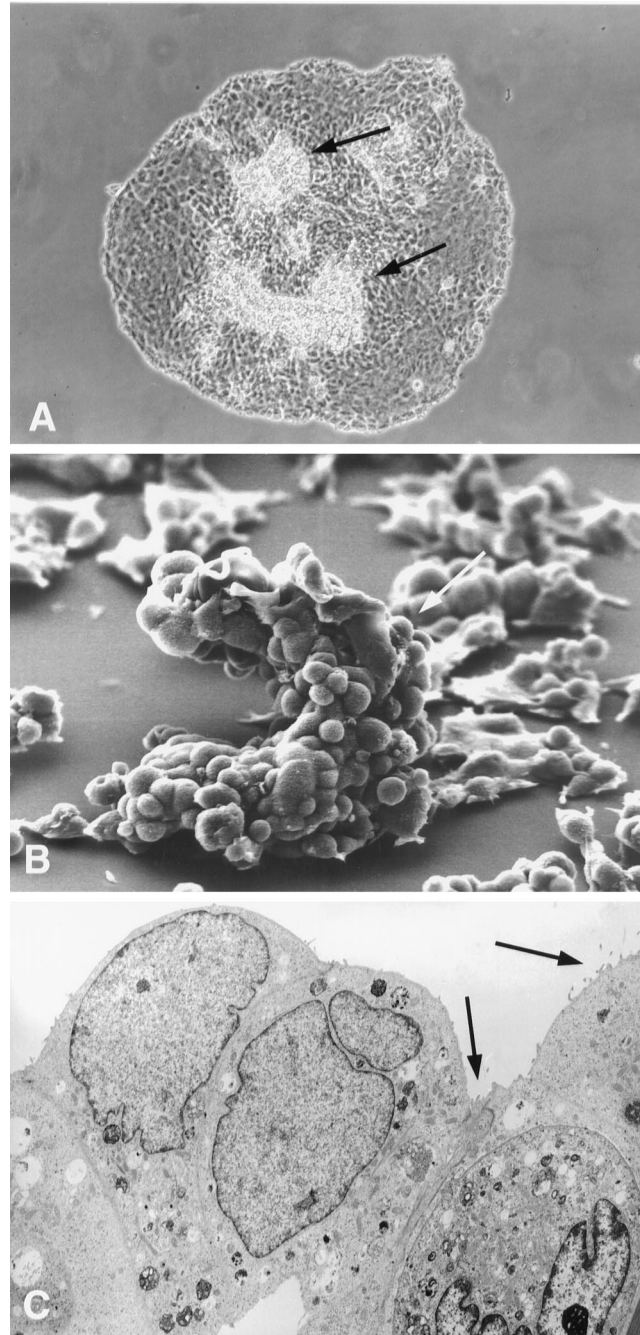


Fig. 4. Growth characteristics of OMIR cells after 5 days of culture in fibronectin-free CM199 (A) or fibronectin-rich SFM (B and C). Tridimensional processes arise from small cell monolayer (A and B, arrows). Microvilli are present in some of the OMIR cells lining these processes (C, arrows). A, $\times 150$; B, $\times 580$; C, $\times 5100$.

Tumorigenicity in Nude Mice. OMIR cells from three different clones, but not OMCs, were able to induce grossly measurable large tumors in nude mice (Fig. 7A). The tumors became palpable as early as 2 weeks after the initial inoculation. One of the clones (clone 8) produced lung metastases. Histologically, the primary tumors and the lung metastases were characterized by highly vascularized and poorly differentiated cells (Fig. 7B) that coexpressed cytokeratin (Fig. 7C) and vimentin (Fig. 7D). Ultrastructurally, the tumor cells exhibited mesothelial-like features including tight junctions, desmosomes, microvilli, and intracellular lumina (Fig. 7E).

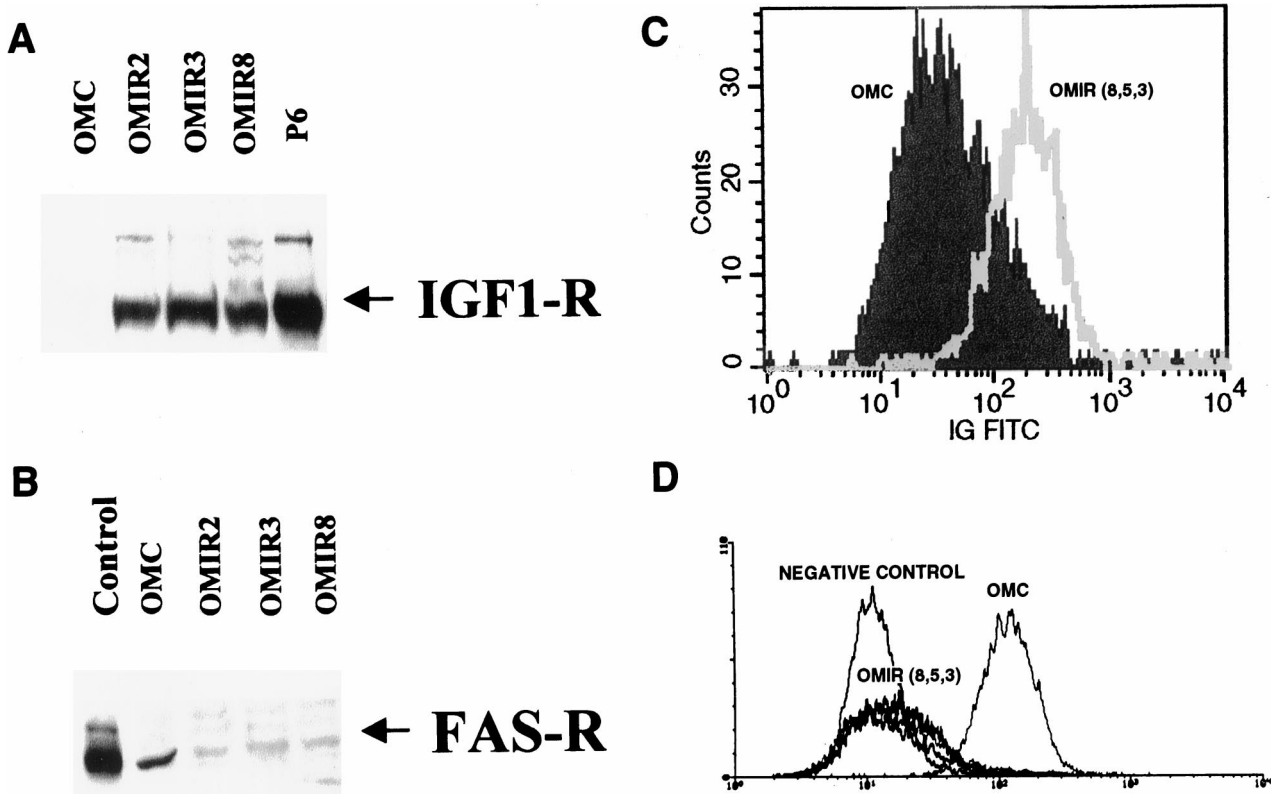


Fig. 5. A, IGF-1R Western blot of OMC and OMIR clones (2, 3, 8). The IGF-1R-positive control is represented by P6 cell [3T3 fibroblasts transfected with a plasmid constitutively expressing IGF-1R; these cells were kindly provided by Dr. Renato Baserga (Jefferson Cancer Center, Philadelphia, PA)]. A protein band of $M_r \sim 135,000$ corresponds to the α subunit of the IGF-1R. B, FasR Western blot of OMC as compared with OMIR clones (2, 3, and 8). The Fas-positive control (Jurkat cells) and OMC, but not the OMIR clones, exhibit a M_r 36,000 Fas protein band. C, flow cytometric evaluation demonstrates that OMIR cells (empty area) express a larger number of IGF-1Rs as compared with OMC (shaded area; approximately 1:100 ratio). Conversely, a larger number of Fas-R is present on OMCs as compared with OMIR cells [approximately 1:10 ratio (D)].

DISCUSSION

In the native ovary, prominent morphogenetic activity leads ovarian mesothelial cells to form mesothelial-lined inclusion cysts, and fibrovascular structures or papillae under physiological and pathological conditions (16). Papillogenesis is exuberant in human ovaries harboring low malignant potential and frankly malignant serous neoplasms. In preliminary experiments, we demonstrated that OM proliferative and morphogenetic changes may be related to underlying ovarian events, including growth factor stimulation (9). In this investigation, we have observed that the introduction of a CVN expression vector carrying the gene for the human IGF-1R into normal rabbit OMCs, transforms these cells (OMIR) *in vitro* and *in vivo*.

The IGF-1 is a progression factor able to bind the IGF-1R with high specificity. The activation of this receptor induces a cascade of intracellular tyrosine phosphorylations that eventually culminate in the activation of transcription factors involved in the synthesis of proliferation-inducing proteins (17–19). Experiments performed on mouse embryo fibroblasts homozygous for the targeted disruption of the IGF-1R gene (*R-* cells), have shown that SV40T antigen, an activated and overexpressed H-ras, or a combination of both, were unable to transform *R-* cells but easily transformed wild-type cells expressing the receptor (*W* cells; 20–22). This inability of *R-* cell to be transformed was promptly corrected by the introduction of a plasmid expressing a wild-type IGF-1R (20–21). The implication of these observations is that the presence of IGF-1R is an obligatory requirement for the establishment and maintenance of the transformed phenotype. We believe that a similar requirement is necessary for the establishment of epithelial ovarian cancer. Preliminary investigations have concluded that the growth of normal and neoplastic ovarian

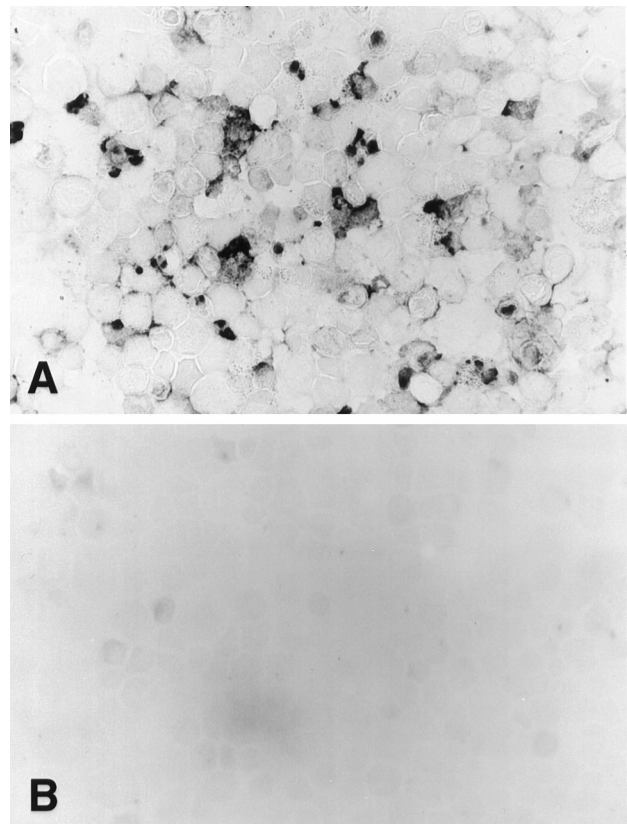


Fig. 6. Apoptotic OMC cells (A) as indicated by the dark nuclear staining. Nuclear staining is absent in CH11-exposed OMIR cells (B). $\times 540$.

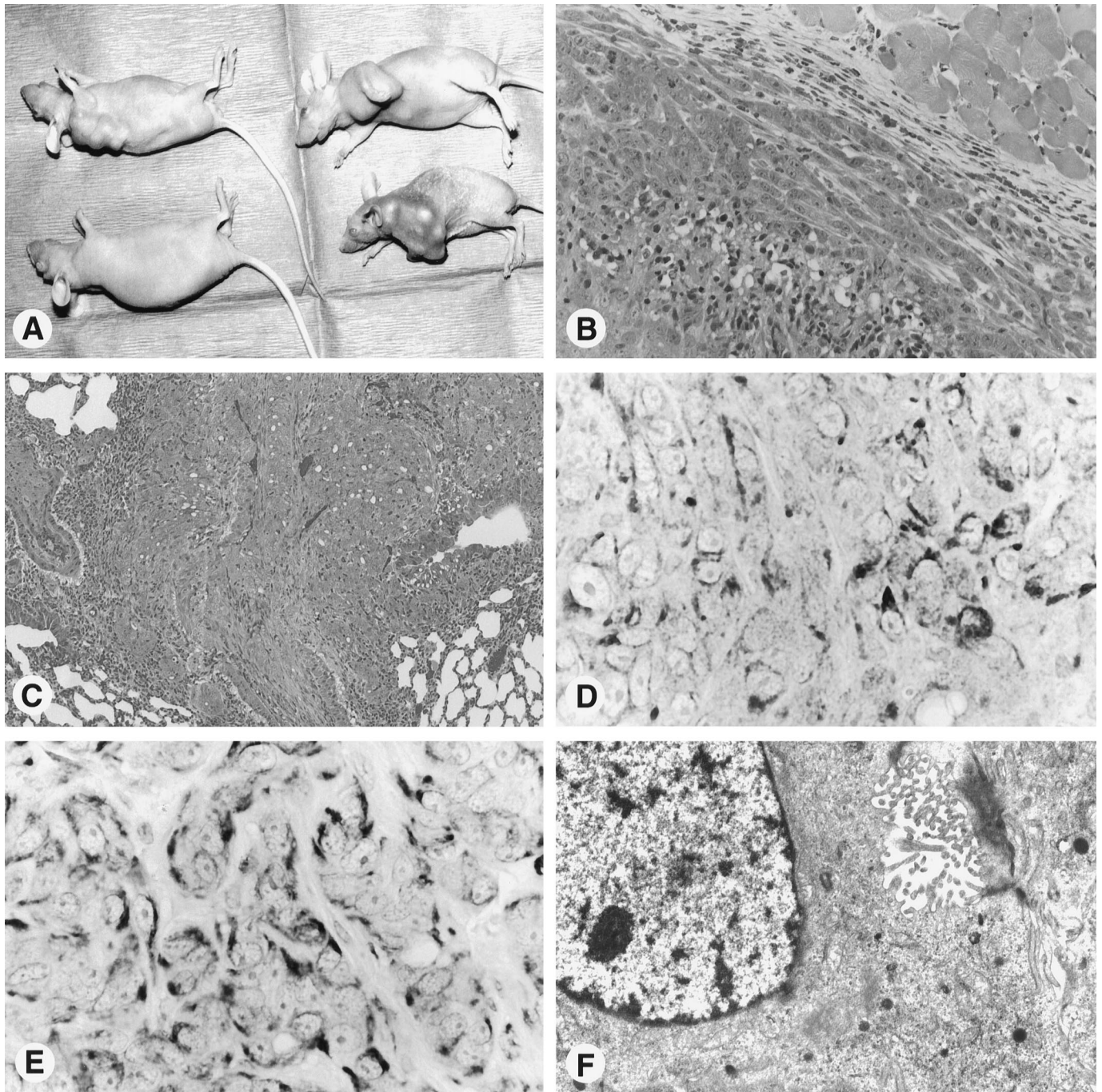


Fig. 7. Disfiguring tumors in BALB/c nude mice 3 weeks after injection with 5×10^6 OMIR cells (clones 2, 3, and 8). In contrast, the nude mouse in the *left lower corner*, free of tumor, had been given injections with OMCs (A). Histologically, the tumors were poorly differentiated, involving skeletal muscle (B). Clone 8-derived tumor metastasized to the lung (C). The tumor cells coexpressed cytokeratin (D) and vimentin (E), and revealed ultrastructural evidence of mesothelial differentiation (F).

mesothelial cells are dependent upon the autocrine stimulation of the IGF-1/IGF-1R system (10, 11, 23, 24). For example, it has recently been shown that IGF-1- and of IGF-1R-binding proteins are expressed in three different ovarian carcinoma cell lines (OVCAR-3, OVCAR-7, and PEO4; Ref. 10). It has also been observed that CAOV-3 and OVCAR-3 cells produce endogenous IGF-1 and grow autonomously in SFM. Their growth in these conditions, however, is further stimulated by the addition of IGF-1 (11). Other investigators confirmed this finding in ovarian- and mesothelial-derived tumors (25, 26). Treatment with IGF-1R mRNA antisense oligodeoxynucleotides markedly inhibits the proliferation of these cells both in SFM and in the presence of IGF-1. Such inhibition corresponds to a reduction in the

amount of detectable phosphorylated IGF-1R (11). Taken together, these data indicate that the IGF-1/IGF-1R system, has a prominent role in the proliferation and transformation of human ovarian mesothelial cells. This hypothesis is validated by our results showing that OMCs, if induced to express a high number of IGF-1Rs, become transformed and tumorigenic. Interestingly, even in SFM alone, OMIR cells proliferated, although at a lower rate (~ 8 -fold), implying the presence of an IGF-1 autocrine loop. The findings of increased IGF-1- and of IGF-1-binding protein 2 in cyst fluid of epithelial ovarian carcinomas also support this contention (27).

OMIR cells, but not OMCs, produced large debilitating tumors in nude mice, which demonstrates their behavioral similarity to carci-

noma cells. The inhibition of growth *in vitro* after treatment with IGF-1R mRNA antisense oligonucleotides indicates that the transformed phenotype is strictly dependent on the overexpression of a functional IGF-1R. These findings are in agreement with the earlier observation of involvement of the IGF-1R in ovarian epithelial carcinogenesis (11).

Recent reports have implicated IGF-1R in programmed cell death (apoptosis; Refs. 12, 13, 28). Apoptosis is a highly ordered process characterized by nuclear changes including chromatin condensation, fragmentation, and internucleosomal DNA cleavage. The biochemical and molecular aspects of apoptosis have recently been delineated (29–31). Resnicoff *et al.* have shown that an activated and overexpressed IGF-1R has a protective role in apoptosis (28), and that this function is independent of its mitogenic action (13). There is evidence that cells may escape death by an IGF-1R-mediated increase in Bcl-2 and Bcl-X_L (32), and/or by a failure of p53 in inhibiting mitogenic signaling by IGF-1R via secretion of IGF-binding proteins (33, 34). However, the complete mechanism by which this action takes place is currently unknown. A possibility is that the activation of IGF-1R modulates changes in the expression of death-inducing signaling complex proteins and/or receptors (MORT/FADD, MACH/FLICE, FAS, FAP-1, etc.). In particular, Fas (APO-1, CD95) is a M_r 48,000 cellular-membrane protein belonging to the tumor necrosis factor receptor family (TNF/NGF; Refs. 29, 30). When activated, this receptor is able to trigger apoptosis. The binding of Fas ligand to Fas-R induces trimerization of the receptor and activation of the death-inducing signaling cascade, which ultimately stimulates the interleukin-1 β -converting enzyme proteases and cell death (31). Fas has been reported in normal but not in malignant ovarian surface epithelium (30). Furthermore, in this study we have observed an inverse relationship between expression of FasR and IGF-1R in OMCs and OMIR cells. FasR was expressed in OMCs but not in OMIR cells, which suggests that the antiapoptotic action of an overexpressed IGF-1R may represent the end result of the modulation of FasR and/or Fas-dependent signaling cascade by the activated and overexpressed IGF-1R. Additional investigations on the interaction between apoptotic and antiapoptotic signals, using OMC/OMIR cells should provide new insights into the pathobiology of the ovarian mesothelium and ovarian epithelial cancer.

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