The Presence of Simian-Virus 40 Sequences in Mesothelioma and Mesothelial Cells Is Associated with High Levels of Vascular Endothelial Growth Factor

Paola Cacciotti, Luigi Strizzi, Giovina Vianale, Laura Iaccheri, Roberta Libener, Camillo Porta, Mauro Tognon, Giovanni Gaudino, and Luciano Mutti

Department of Medical Sciences, University of Piemonte Orientale "A. Avogadro," Novara: Department of Oncology and Neurosciences, "G. D'Annunzio" University, Chieti; ASL-11, Piemonte and IRCSS Maugeri Fondation, Pavia; Department of Morphology and Embryology, Section of Histology and Embryology, University of Ferrara, Ferrara; Pathology Service, SS. Antonio e Biagio Hospital, Alessandria; and IRCCS, San Matteo University Hospital, Pavia, Italy

The aim of this study was to evaluate whether the presence of simian virus-40 (SV40) is associated with increased release of vascular endothelial growth factor (VEGF) in human malignant mesothelioma (MM) cells. We studied nine cell lines derived from pleural effusion (PE) of patients with MM, and three different cultures of normal human mesothelial cells (NHMC) derived from pleural fluid of patients with congestive heart failure. NHMC were transfected with full length SV40 (NHMC-FL) or large T antigen (NHMC Tag) DNAs. High levels of VEGF were detected in conditioned media of each of two MM cells that tested positive for SV40 by PCR amplification and Southern blot hybridization and for Tag transcript by reverse transcription–polymerase chain reaction (RT-PCR) and immunoprecipitation. We also found that NHMC-FL released high amounts of VEGF. Conditioned media from SV40-positive MM cells and from FL-NHMC increased proliferation of human umbilical vein cells (HUVEC) and this effect was partially abrogated by adding specific blocking antibodies against VEGF. These results offer the first evidence that SV40 can cause VEGF release in SV40-positive MM cells and that entire viral genome is required for this effect.

Human malignant pleural mesothelioma (MM), considered a rare human tumor, is showing a dramatic increase in incidence worldwide (1, 2). Epidemiologic studies have predicted that approximately a quarter of a million Europeans will develop MM during the next few decades and that the frequency of this tumor in the United States has increased by ninety percent in the last twenty years (3, 4). The highly aggressive nature of MM and its resistance to present therapeutic strategies explain why the average survival of most affected patients is only 8–12 mo from diagnosis (5). Comprehension of the pathogenesis and molecular pathways involved in the development of MM will determine whether efficient diagnostic and therapeutic protocols will successfully meet the clinical needs in future cases.

Asbestos is commonly considered by far the most relevant causative agent of MM, although only a fraction of exposed subjects eventually develop the disease (6). Numerous studies have shown the expression of Simian Virus 40 (SV40) in MM tissue (7–9) and that SV40 viral gene product, Tag, binds and inactivates cell cycle regulatory proteins like p53 and Rb (10, 11). In addition, SV40 is capable of replication in mesothelial cells (12, personal communication), and SV40 sequences in nonepithelial MM correlates with poor prognosis (13). All this evidence suggests that further studies are needed to determine the role of this highly oncogenic DNA virus in MM pathogenesis.

Different growth factors, like platelet-derived growth factor A and B (14), insulin-like growth factor-1 (IGF-1) (15), basic-fibroblast growth factor (b-FGF) (16), and hepatocyte growth factor (HGF) (17) have been shown to play a significant role in the development and progression of MM. Correlation between diminished patient survival and increased expression of vascular endothelial growth factor (VEGF) or b-FGF has been recently confirmed in patients with MM (18, 19). In particular, it was suggested that in addition to potential angiogenic properties, VEGF might contribute to decreased MM patient survival by directly stimulating the growth of MM cells in an autocrine fashion (18). We found that the production of HGF in normal and malignant mesothelial cells was increased after SV40 transfection and that HGF synthesis and HGF-dependent autocrine stimulation may lead to a growth advantage in SV40-positive mesothelial cells (personal communication). Interestingly, a recent report also showed that the release of HGF was capable of regulating VEGF and VEGF receptor expression (20).

In light of these findings, our study is aimed at investigating whether SV40 status may affect VEGF expression in normal human mesothelial (NHMC) and MM cells. For this purpose we measured the levels of VEGF in conditioned media of primary MM cell cultures derived from SV40-positive and -negative patients with MM. We also investigated whether transfection of SV40 DNA or Tag DNA into primary NHMC could influence VEGF expression in culture media. Finally, we indirectly assessed the angiogenic potential in SV40-positive MM by determining cell proliferation of human umbilical vein endothelial cells (HUVEC) cultured with conditioned media (CM) from SV40-positive MM cells and SV40-transfected NHMC (NHMC-FL).

Materials and Methods

Cell Lines

Primary MM cell cultures and primary NHMC were derived from pleural fluids following previously described methods (15,

(*Received in original form July 10, 2001 and in revised form October 18, 2001*)

Address correspondence to: Dr. Luciano Mutti, Ospedale S. Pietro e Paolo, ASL-11 Vercelli, Piazzale Lora, 1, 13011 Borgosesia (VC), Italy. E-mail: ftepjm@tin.it

Abbreviations: conditioned media, CM; human umbilical vein cells, HUVEC; L-glutamine, L-Glu; malignant mesothelioma, MM; normal human mesothelial cells, NHMC; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; pleural effusion, PE; reverse transcription-PCR, RT-PCR; simian virus-40, SV40; vascular endothelial growth factor, VEGF.

Am. J. Respir. Cell Mol. Biol. Vol. 26, pp. 189–193, 2002
Internet address: www.atsjournals.org

21). MM cells were separated from the pleural effusion (PE) of nine patients with MM, while NHMC were isolated from three patients with transudative pleural fluid due to congestive heart failure and with no history of malignant disease. The mesothelial origin of the isolated cells was confirmed by positive immunostaining with commercially available antibodies raised against cytokeratins, vimentin, or calretinin, as previously described (21). Furthermore, negative immunostaining with antibodies against carcinoembryonic antigen (CEA), Leu-M1, and B72.3 ruled out the epithelial origin of these cells. Cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin (PS), 1% L-glutamine (L-Glu) (GIBCO BRL Life Technologies Inc., Gaitersburg, MD) and maintained in a humidified atmosphere at 37°C and 5% CO_2.

Transfection with SV40 and Tag DNAs

The three primary NHMC cultures were transfected with either SV40 full length DNA (NHMC-FL) or with the recombinant vector pSV3neo expressing SV40 Tag (NHMC-Tag). Nontransfected NHMC or NHMC transfected with irrelevant pSV3neo plasmid (NHMC-IR) were used as negative controls. Stable transfectants were obtained by transfection of pSV3neo and SV40 DNA using the polycation compound "Superfect" as indicated by the supplier (Qiagen GmbH, Hilden, Germany). Growth selection was performed in growth medium supplemented with 0.8mg/ml G418-sulfate (Geneticin; GIBCO BRL Life Technologies Inc.). Stable cell cultures were examined by immunoblotting using a monoclonal antibody to Tag (Ab-1; Oncogene Science, Tarrytown, NY).

Polymerase Chain Reaction, Reverse Transcription–Polymerase Chain Reaction, and Hybridization for Detection of SV40 Sequences

Amplification of 250 ng genomic DNA was performed by polymerase chain reaction (PCR). Cycling parameters were: 1 min at 95°C, 30 s at 56°C, and 16 s at 72°C for 35 cycles. Approximately 400 ng of total RNA, extracted with the guanidinium thiocyanate system (Rneasy Kit, Quiagen GmbH), were used for reverse transcription (RT)-PCR (Accept RT-PCR Kit; Promega Biotec, Madison, WI). The reaction mixture was incubated at 48°C for 45 min and after heat inactivation of AMV-RT at 95°C for 2 min, SV40 cDNA was amplified as follows: 45 s at 95°C, 1 min at 60°C, 1 min at 72°C for 35 cycles with a final extension at 72°C for 10 min. The oligomers used for PCR and RT-PCR amplifications were: SV5: 5′-TAGGTGCCAACCTATGGAACAGA-3′ and SV6: 5′-GAAAGTCTTTAGGGTCTTCTACC-3′.

For the hybridization of PCR products, DNA was transferred from agarose gel to a nylon membrane (Hybond-N+; Amersham Pharmacia, Little Chalfont, UK) and UV cross-linked. The filters were exposed to pre-hybridization solution, saturated with denatured salmon sperm DNA (Sigma Aldrich, Milan, Italy) and incubated with the labeled probe (region 4402–4570 of the SV40 genome) overnight at 60°C. The probe was radiolabeled with 10 μl [α-^{32}P]-dCTP (specific activity 3000 Ci/mmol; Amersham) using the Random-Primed Kit (Boehringer Mannheim, Milan, Italy) and unincorporated nucleotides were removed on Quick Spin columns (Sephadex G-50; Boehringer Mannheim). Nonspecific binding was removed by washing the nylon filter with wash buffer 1 (0.01% SDS–10% SSC) and wash buffer 2 (0.01% SDS–0.1% SSC) at 60°C.

Immunoprecipitation and Immunoblotting for Detection of SV40 Tag Protein

Cells were washed with cold phosphate-buffered saline (PBS) and lysed with ice-cold Rapid Immunoprecipitating Agent (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% TRITON X-100, 0.5% sodium deoxycholate, 5 mM EDTA) buffer containing protease inhibitors (aprotinin, 10 mg/ml; pepstatin, 10 mg/ml; leupeptin, 50 mg/ml; phenylmethylsulfonyl fluoride, 1mM). Cell lysates were cleared by centrifugation. An equal amount (800 μg) of total protein was immunoprecipitated by gentle mixing for 2 h at 4°C with anti-Tag (Ab-1; Oncogene Science) specific antibody (200 ng/ml) adsorbed to 40 μl protein A-Sepharose beads (Amersham Pharmacia). Immunocomplexes were washed six to eight times with lysis buffer and proteins were dissociated from immunoprecipitates by boiling in Laemmli buffer in reducing conditions. Proteins were separated on SDS-7.5% polyacrylamide gels and transferred to nitrocellulose filters (Hybond; Amersham). Filters were probed with the same antibody used for immunoprecipitation and specific binding was detected by the enhanced chemiluminescence system (ECL; Amersham).

VEGF Levels

Immunoassay of VEGF was performed on conditioned medium (CM) from subconfluent NHMC, NHMC-Tag, NHMC-FL, NHMC-IR, and from SV40-positive and SV40-negative MM PE and primary cell cultures using a commercial ELISA kit and following manufacturer's instructions (R&D Systems, Abingdon, UK).

Effect of Conditioned Medium from SV40-Positive MM Cells and NHMC-FL on HUVEC Growth

The potential angiogenic effect of SV40-positive MM was tested by incubating HUVEC with culture medium (Medium 199, 2% L-Glu, 1% PS, 0.2% Gentamycin, 10% heparin, 10% ECGF, 20% fetal calf serum; GIBCO BRL Life Technologies Inc.) supplemented with CM (1:1) obtained from a representative SV40-positive MM patient and NHMC-FL. After 24 and 48 h of growth, HUVEC proliferation was evaluated by microscopic observation and quantified by cell counts using the trypan-blue exclusion method. Cell counts were then compared with control HUVEC, HUVEC grown in culture medium containing 10 ng/ml of rhVEGF (R&D Systems), and HUVEC grown in culture medium containing CM pretreated with 40 ng/ml of anti-VEGF blocking antibodies (R&D Systems). The specificity and neutralization characteristics of the blocking VEGF antibody was previously tested and described by the manufacturer.

Statistics

All assays and cell counts were performed in triplicate and expressed as mean value ± SD. Students' t test was employed to evaluate statistical significance of the differences in mean value ± SD between groups. Statistically significant findings were considered when $P < 0.05$.

Results

Detection of SV40 Sequences and Tag Expression

We examined cell lines derived from the PE of nine individuals with MM. PCR and hybridization analysis of genomic DNA performed on MM cells using SV5 and SV6 primers, encompassing nucleotides 4402–4574 of the SV40 genome (7), revealed the presence of SV40 early region DNA in two cell lines (MMP and MMCa). These viral sequences were transcriptionally active, because Tag expression was observed by RT-PCR and immunoblotting in these same cells. Tag transcript was not detected in the other seven cell lines. Cell morphology referring to the histologic subtypes of the original tumor did not reveal any association with the presence of the SV40 sequences (Table 1).

TABLE 1
Characteristics of cell lines studied and histotypes related

Cell lines	sV40-Tag Sequence*	Tag Expression†	Cell Morphology‡
Control (COS-7)	+	+	Fibroblastoid
MMB	−	−	Epithelial
MMC	−	−	Epithelial
MMA	−	−	Sarcomatous
MMCa	+	+	Epithelial
MMP	+	+	Sarcomatous
MMM	−	−	Sarcomatous
MMO	−	−	Sarcomatous
MMG	−	−	Epithelial
MMCo	−	−	Epithelial
NHMC A	−	−	Epithelioid
NHMC A-Tag	+	+	Fibroblastoid
MHMC A-FL	+	+	Fibroblastoid
NHMC B	−	−	Epithelioid
NHMC B-Tag	+	+	Fibroblastoid
NHMC B-FL	+	+	Fibroblastoid
NHMC C	−	−	Epithelioid
NHMC C-Tag	+	+	Mixed
NHMC C-FL	+	+	Fibroblastoid

Definition of abbreviations: MM, malignant mesothelioma; NHMC, normal human mesothelial cells.
* PCR (SV5-SV6 primers).
†RT-PCR and immunoblotting.
‡Histologic subtypes of MM cell lines and morphologic appearance of NHMC before and following transfection (cytokeratin, vimentin, and calretinin markers).

The three NHMC primary cultures were transfected with either SV40 DNA (NHMC-FL) or with pSV3neo expressing Tag (NHMC-Tag). PCR analysis of G418 selected clones confirmed the presence of SV40 Tag encoding sequence. Immunoprecipitation and immunoblotting performed on selected clones showed that both types of transfectants displayed expression of SV40 Tag (Table 1).

Evaluation of VEGF Release

Figure 1 shows mean VEGF levels measured in CM from the different cell lines tested. Mean VEGF levels were significantly higher in SV40-positive primary MM cell cultures (1579 ± 367 pg/ml) and in NHMC-FL (1554 ± 312 pg/ml) compared with the mean level measured in CM of SV40-negative MM cells (307 ± 102 pg/ml) ($P < 0.01$), NHMC (168 ± 27) ($P < 0.01$), NHMC-IR (668 ± 75) ($P < 0.05$), and NHMC-Tag (815 ± 53) ($P < 0.05$). The mean VEGF level in CM of NHMC-Tag was significantly higher than either SV40-negative MM ($P < 0.01$) or NHMC ($P < 0.001$). Mean VEGF level in CM of NHMC-Tag was greater than the mean level found in NHMC-IR, though the difference was at the limit of statistical significance ($P = 0.05$).

HUVEC Proliferation

After 48 h, cellular density evaluated by light microscopic observation was more evident in HUVEC grown in medium supplemented with CM of SV40-positive MM cells compared with controls. In particular, compared with controls, cell counts significantly increased after 24 h ([558 ± 42] × 10^3 cells) and 48 h ([710 ± 92] × 10^3 cells) of growth with CM of SV40-positive MM cells ($P < 0.01$) or from

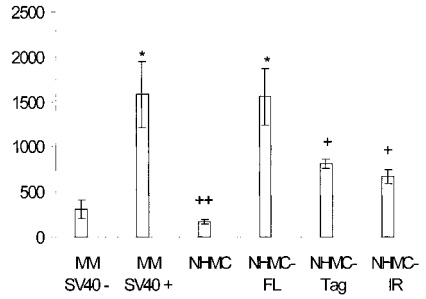

Figure 1. Mean VEGF levels measured in CM from different MM cell lines tested. Mean VEGF level in CM of NHMC-Tag was greater than the mean level found in NHMC-IR, though the difference was at the limit of statistical significance ($P = 0.05$). $^*P < 0.01$; $^+P < 0.05$; $^{++}P < 0.05$.

NHMC-FL (Figure 2). The significant trend was observed after 24 h ([540 ± 42] × 10^3 cells) and 48 h ([640 ± 76] × 10^3 cells) ($P = 0.01$) with HUVEC grown in control medium supplemented with rhVEGF. The addition of blocking antibodies against human VEGF to the culture medium containing CM from MM cells did not significantly affect cell proliferation at 24 h ([478 ± 64] × 10^3 cells) and 48 h ([574 ± 98] × 10^3 cells) ($P > 0.05$).

Discussion

In this study, high VEGF levels were detected for the first time in CM of SV40-positive primary MM cell cultures. These levels were significantly higher compared with the levels measured in growth medium of SV40-negative MM cell cultures. Also, transfection of NHMC with full length SV40 DNA resulted in a significant increase of VEGF levels in CM compared with the levels found in CM of non-transfected NHMC and in NHMC-Tag. These results suggest that SV40 may participate in determining increased VEGF production by mesothelial cells.

These observations are intriguing in the context of recent reports showing that SV40 replication can occur in human mesothelial cells (12) and that a VEGF-dependent autocrine circuit can sustain MM cell growth *in vitro* (18). In light of these observations, it is possible to speculate that SV40 may determine a growth advantage in SV40-positive MM cells, not only by inactivating cell cycle regulatory proteins, as previously suggested (10, 11), but also by inducing increased expression of specific growth factors, including VEGF. The latter hypothesis is supported by findings in an animal model, where high growth rate and *in vivo* tumorigenicity were shown to be associated with increased IGF-1 release in SV40-positive MM tumor cells (22).

How SV40 may be involved in growth factor–regulatory mechanisms is unclear. It can be speculated as to whether specific region(s) of the SV40 genome can act as a transcription factor for the synthesis of growth factors, like IGF-1 or VEGF. Future investigation employing specifically engineered defective SV40 viruses would be necessary to investigate this possibility.

Inactivation of p53 in breast cancer cells was shown to be associated with decreased expression of thrombospon-

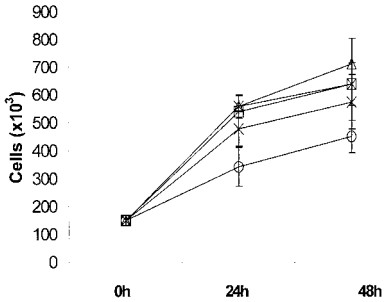

Figure 2. Cell counts, compared with controls, significantly increased after 24 and 48 h of growth with CM of SV40-positive MM cells ($P < 0.01$). The same significant increase was observed after 24 and 48 h ($P = 0.01$) with HUVEC grown in control medium supplemented with rhVEGF. The addition of blocking antibodies against human VEGF to the culture medium containing CM from MM cells did not significantly affect cell proliferation at 24 and 48 h ($P > 0.05$). *Circles*, control HUVEC; *squares*, HUVEC + rhVEGF ($P = 0.01$); *triangles*, HUVEC + MM CM ($P < 0.01$); ×, HUVEC + anti-VEGF ($P > 0.05$); *six-armed crosses*, NHMC-FL.

din-1, a well known extracellular matrix protein capable of regulating endothelial growth by antagonizing VEGF pro-angiogenic effects (23). Because SV40 Tag protein has been shown to bind and inactivate p53 (10), this mechanism may indirectly favor increased VEGF production in MM and can explain why our SV40-positive MM cells produce higher VEGF levels. Another possibility derives from our recent observations of increased HGF production and Met activation in mesothelial cells transfected with SV40 DNA (personal communication). Interestingly, HGF has been linked with increased VEGF production and flk-1 expression (20). Whether significant VEGF production is a consequence of SV40-induced HGF activity in MM needs to be further studied.

The findings described here show that NHMC-FL release greater quantities of VEGF compared with NHMC-Tag, suggesting that VEGF release in NHMC is related to the presence of the entire SV40 genome. This difference in VEGF release may be explained by the observation demonstrating cooperation between Tag and small t antigen (tag) for entire SV40-dependent oncogenic effects in human cells (24, 25). The transfection of NHMC with the irrelevant plasmid also caused increased expression of VEGF. This suggests that SV40-dependent transforming effect or malignant status may not be a prerequisite for increased mesothelial cell VEGF production, and that any disturbance of normal mesothelial cell homeostasis may result in increased growth factor production. In fact, non-malignant pleural effusions, like empyema, parainfectious effusions, or rheumatoid effusions, have shown higher levels of VEGF compared with noninflammatory pleural transudates (26), and the inoculation of irritants such as asbestos fibers into normal mesothelial lining of experimental animals resulted in increased local angiogenesis, probably secondary to increased production of angiogenic factors (27).

Several studies have reported significantly higher levels of VEGF in malignant compared with nonmalignant pleural effusions (26, 28). In addition, increased tumor vessel density (29–31) and serum VEGF levels (18) were found to correlate with diminished MM patient survival. However, these studies reported contrasting data as to whether tumor vessel formation in MM is dependent on VEGF expression. Because the presence of SV40 has been suggested to represent a negative prognostic factor in MM patients (13), it would be interesting to study, in a larger series, whether poor prognosis caused by increased VEGF levels may reflect SV40 status in MM.

We show that when CM from SV40-positive MM cells is added, the proliferation of HUVEC is stimulated in the same way as control medium containing rhVEGF. Blocking antibodies against human VEGF added to SV40-positive MM CM did not show any significant reduction in HUVEC counts. This suggests that VEGF may not be the only growth factor produced by MM cells capable of stimulating endothelial cell growth, and explains why VEGF-blocking antibodies do not completely interfere with HUVEC proliferation. This is not surprising, because numerous endothelial growth factors, including b-FGF, PDGF, IL-8, and HGF have been detected in CM of MM (32, 33). It has to be recalled that HGF itself is an angiogenetic factor (34) and that it was detected in CM of NHMC-FL (personal communication). As suggested for other cell types (20), the expression of HGF may cause enhancement of VEGF and flk-1 expression. In fact, we found that HUVEC grown with CM from NHMC-FL also produced increased proliferation.

Our findings describe a new mechanism by which SV40 might favor MM proliferation. As our MM cells were directly derived from the PE of patients with MM, these data suggest that the described mechanism can occur *in vivo* as well. Indeed, SV40 could promote MM growth by stimulating, through still unknown mechanisms, the production of VEGF. Because VEGF appears to play an important role in MM proliferation by stimulating autocrine growth of tumor cells, our data support the rationale for addressing future research toward experimental clinical trials employing anti-angiogenic agents for the treatment of MM.

Acknowledgments: The authors thank the Italian Group for Study and Therapy of Malignant Mesothelioma (G.I.M.E.) for their encouragement and support. This study was supported in parts by the Italian Ministry of University, Research and Technology (MURST) PRIN grants (M.T. and G.G.), Italian Association for Cancer Research (AIRC) grants (M.T. and G.G.), and National Research Council, Biotechnology Target Project grants (M.T. and G.G.).

References

1. Antman, K. H. 1993. Natural history and epidemiology of malignant mesothelioma. *Chest* 103:373S–376S.
2. Carbone, M., S. Fisher, A. Powers, H. I. Pass, and P. Rizzo. 1999. New molecular and epidemiological issues in mesothelioma: role of SV40. *J. Cell. Physiol.* 180:167–172.
3. Fisher, S., L. Weber, and M. Carbone. 1999. Cancer risk associated with simian virus 40 contaminated poliovaccine. *Anticancer Res.* 19:2173–2180.
4. Peto, J., A. Decarli, C. La Vecchia, F. Levi, and E. Negri. 1999. The European mesothelioma epidemic. *Br. J. Cancer* 79:666–672.
5. Boutin, C., M. Schlesser, C. Frenay, and P. Astoul. 1998. Malignant pleural mesothelioma. *Eur. Respir. J.* 12:972–981.
6. de Klerk, N. H., and B. K. Armstrong. 1992. The epidemiology of asbestos and mesothelioma. *In* D. W. Henderson, K. B. Shilkin, S. Langlois, and D. Whitaker, editors. Malignant Mesothelioma. Hemisphere Publishing Corp, New York. 223–252.
7. Carbone, M., H. I. Pass, P. Rizzo, M. Marinetti, M. Di Muzio, D. J. Mew, A. S. Levine, and A. Procopio. 1994. Simian virus 40-like DNA sequences in human pleural mesothelioma. *Oncogene* 9:1781–1790.

8. Shivapurkar, N., T. Wiethege, I. I. Wistuba, E. Salomon, S. Milchgrub, K. M. Muller, A. Churg, H. Pass, and A. F. Gazdar. 1999. Presence of simian virus 40 sequences in malignant mesotheliomas and mesothelial cell proliferations. *J. Cell Biochem.* 76:181–188.
9. Strizzi, L., G. Vianale, M. Giuliano, R. Sacco, F. Tassi, P. Chiodera, P. Casalini, and A. Procopio. 2000. SV40, JC and BK expression in tissue, urine and blood samples from patients with malignant and nonmalignant pleural disease. *Anticancer Res.* 20:885–890.
10. Carbone, M., P. Rizzo, P. M. Grimley, A. Procopio, D. J. Mew, V. Shridhar, A. de Bartolomeis, V. Esposito, M. T. Giuliano, S. M. Steinberg, A. S. Levine, A. Giordano, and H. I. Pass. 1997. Simian virus-40 large-T antigen binds p53 in human mesotheliomas. *Nature Med.* 3:908–912.
11. De Luca, A., A. Baldi, V. Esposito, C. M. Howard, L. Bagella, P. Rizzo, M. Caputi, H. I. Pass, G. G. Giordano, F. Baldi, M. Carbone, and A. Giordano. 1997. The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. *Nature Med.* 3:913–916.
12. Bocchetta, M., I. Di Resta, A. Powers, R. Fresco, A. Tosolini, J. R. Testa, H. I. Pass, P. Rizzo, and M. Carbone. 2000. Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity. *Proc. Natl. Acad. Sci. USA* 97:10214–10219.
13. Procopio, A., L. Strizzi, G. Vianale, P. Betta, R. Puntoni, V. V. Fontana, G. Tassi, F. Gareri, and L. Mutti. 2000. Simian virus-40 sequences are a negative prognostic cofactor in patients with malignant pleural mesothelioma. *Genes Chromosomes Cancer* 29:173–179.
14. Versnel, M. A., A. Hagemeijer, M. J. Bouts, T. H. van der Kwast, and H. C. Hoogsteden. 1988. Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* 2:601–605.
15. Lee, T. C., Y. Zhang, C. Aston, R. Hintz, J. Jagirdar, M. A. Perle, M. Burt, and W. N. Rom. 1993. Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor I and associated molecules. *Cancer Res.* 53:2858–2864.
16. Asplund, T., M. A. Versnel, T. C. Laurent, and P. Heldin. 1993. Human mesothelioma cells produce factors that stimulate the production of hyaluronan by mesothelial cells and fibroblasts. *Cancer Res.* 53:388–392.
17. Harvey, P., A. Warn, S. Dobbin, N. Arakaki, Y. Daikuhara, M. C. Jaurand, and R. M. Warn. 1998. Expression of HGF/SF in mesothelioma cell lines and its effects on cell motility, proliferation and morphology. *Br. J. Cancer* 77:1052–1059.
18. Strizzi, L., A. Catalano, G. Vianale, S. Orecchia, A. Casalini, G. Tassi, R. Puntoni, L. Mutti, and A. Procopio. 2001. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. *J. Pathol.* 193:468–475.
19. Strizzi, L., G. Vianale, A. Catalano, R. Muraro, L. Mutti, and A. Procopio. 2001. Basic fibroblast growth factor in mesothelioma pleural effusions: correlation with patient survival and angiogenesis. *Int. J. Oncol.* 18:1093–1098.
20. Wojta, J., C. Kaun, J. M. Breuss, Y. Koshelnick, R. Beckmann, E. Hattey, M. Mildner, W. Weninger, T. Nakamura, E. Tschachler, and B. R. Binder. 1999. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. *Lab. Invest.* 79:427–438.
21. Orengo, A. M., L. Spoletini, A. Procopio, R. E. Favoni, A. De Cupis, A. Ardizzoni, B. Castagneto, M. Ribotta, P. G. Betta, S. Ferrini, and L. Mutti. 1999. Establishment of four new mesothelioma cell lines: characterization by ultrastructural and immunophenotypic analysis. *Eur. Respir. J.* 13:527–534.
22. Pass, H. I., D. J. Mew, M. Carbone, W. A. Matthews, J. S. Donington, R. Baserga, C. L. Walker, M. Resnicoff, and S. M. Steinberg. 1996. Inhibition of hamster mesothelioma tumorigenesis by an antisense expression plasmid to the insulin-like growth factor-1 receptor. *Cancer Res.* 56:4044–4048.
23. Roberts, D. D. 1996. Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10:1183–1191.
24. Rundell, K., S. Gaillard, and A. Porras. 1998. Small-t and large-T antigens cooperate to drive cell proliferation. *Dev. Biol. Stand.* 94:289–295.
25. Mungre, S., K. Enderle, B. Turk, A. Porras, Y. Q. Wu, M. C. Mumby, and K. Rundell. 1994. Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. *J. Virol.* 68:1675–1681.
26. Thickett, D. R., L. Armstrong, and A. B. Millar. 1999. Vascular endothelial growth factor (VEGF) in inflammatory and malignant pleural effusions. *Thorax* 54:707–710.
27. Branchaud, R. M., J. L. MacDonald, and A. B. Kane. 1989. Induction of angiogenesis by intraperitoneal injection of asbestos fibers. *FASEB J.* 6:1747–1752.
28. Cheng, D., R. M. Rodriguez, E. A. Perkett, J. Rogers, G. Bienvenu, U. Lappalainen, and R. W. Light. 1999. Vascular endothelial growth factor in pleural fluid. *Chest* 116:760–765.
29. Kumar-Singh, S., P. B. Vermeulen, J. Weyler, K. Segers, B. Weyn, A. Van Daele, L. Y. Dirix, A. T. Van Oosterom, and E. Van Marck. 1997. Evaluation of tumour angiogenesis as a prognostic marker in malignant mesothelioma. *J. Pathol.* 182:211–216.
30. Ohta, Y., V. Shridhar, R. K. Bright, G. P. Kalemkerian, W. Du, M. Carbone, Y. Watanabe, and H. I. Pass. 1999. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br. J. Cancer* 81:54–61.
31. Konig, J. E., E. Tolnay, T. Wiethege, and K. M. Muller. 1999. Expression of vascular endothelial growth factor in diffuse malignant pleural mesothelioma. *Virchows Arch.* 435:8–12.
32. Garlepp, M. J., and C. C. Leong. 1995. Biological and immunological aspects of malignant mesothelioma. *Eur. Respir. J.* 8:643–650.
33. Xin, X., S. Yang, and G. Ingle. 2001. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis *in vitro* and *in vivo*. *Am. J. Pathol.* 158:1111–1120.
34. Bussolino, F., M. F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, and P. M. Comoglio. 1992. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.* 119:629–641.