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Spreading of Mesothelioma Cells Is Rapamycin-Sensitive and Requires Continuing Translation

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ABSTRACT

The interaction of cancer cells with extracellular matrix (ECM) is important in metastasization. Here we identified the molecules of the ECM expressed by sarcomatous malignant mesothelioma, and their effect on adhesion and spreading. In addition, by analyzing the relationship between translation and attachment to matrix, we found that mesothelioma cells rely on continuing translation to efficiently attach to matrix, and rapamycin inhibition affects spreading and migration of cancer cells. Specifically, we found that sarcomatous cells produce high amounts of fibronectin, able to support the spreading of mesothelioma cells. Spreading of cancer cells on fibronectin does not require de novo transcription but is sensitive to cycloheximide, an inhibitor of protein synthesis. Next, we analyzed the involvement of the mammalian target of rapamycin (mTOR) pathway, a major pathway controlling translation. Cancer cells have a constitutively active mTOR pathway; surprisingly, inhibition of mTOR complex 1 (mTORC1) by rapamycin barely affects the global rate of translation and of initiation of translation, but deeply inhibits mesothelioma spreading on ECM. The effects of rapamycin and cycloheximide on spreading were observed in several mesothelioma cell lines, although with different magnitude. Overall, data suggest that adhesion and spreading of mesothelioma cells on ECM require the translation of pre-synthesized mRNAs, and mTORC1 activity. We speculate that mTORC1 activity is required either for the translation of specific mRNAs or for the direct modulation of cytoskeletal remodeling. J. Cell. Biochem. 108: 867–876, 2009.

KEY WORDS: INITIATION; FIBRONECTIN; MESOTHELIOMA; mTOR

Malignant mesothelioma (MM) is a highly lethal tumor arising from the mesothelium of the serous cavities. The risk or incidence of MM shows a peculiar dose–response relation to cumulative asbestos exposure. Until recently, nearly all patients succumbed to this tumor within 4–18 months after diagnosis [Goudar, 2007; Fennell et al., 2008], especially due to the involvement of vital thoracic organs and structures. However, recent advances in MM treatment, including debulking surgery, have made the control of the local disease possible, thus revealing an increasing clinical importance of the metastatic disease. Metastasization in MM occurs by direct spread as well as through lymphatics and more rarely the bloodstream. Invasion by seeding of the needle biopsy tract or the thoracoscopy/thoracotomy incision site is also a common occurrence related to the diagnostic procedures.

Mesothelioma are classified as epithelial, sarcomatous, or biphasic according to their histological appearance. The sarcomatous type is the less frequent, but the most aggressive and rapidly fatal. The invasive behavior of sarcomatous MM cells taken together

Abbreviations used: MM, malignant mesothelioma; ECM, extracellular matrix; mTOR, mammalian target of rapamycin; CM, conditioned medium; DMEM; Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline solution; FITC-phalloidin, fluorescein isothiocyanate-conjugated phalloidin; Fn, fibronectin.

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with the production of extracellular matrix (ECM) factors [Klominek et al., 1993] suggests that the local synthesis of ECM components may facilitate local tumor spread. Therefore, it would be important to identify the ECM proteins produced by sarcomatous MM cells in order to improve our understanding of their mechanisms of invasiveness.

ECM factors and their receptors, integrins, have been involved in the MM cell adhesion and migration. For instance, fibronectin can induce the haptotactic migration of MM cells [Klominek et al., 1997]. In addition, matrix metalloproteases have been involved in the capacity of MM cells to digest the surrounding matrix [Zhong et al., 2006]. However, it is still unclear whether MM cells produce ECM substances that directly promote their attachment and survival, and if so, which molecular mechanisms regulate the early biochemical events following the adhesion of tumor cells to the matrix. In this study, we have addressed this issue, and identified ECM molecules secreted by MM cells, and the biochemical events that regulate adhesion and spreading.

Sarcomatous and epithelial MM cell lines were used to study their capacity to promote adhesion to the matrix, spreading, and migration. We found that the conditioned medium (CM) from a sarcomatous line supports adhesion and spreading due to the presence of fibronectin. We found that tumor cells do not require fibronectin to sustain mammalian target of rapamycin (mTOR) activation and translation. Rather, inhibition of translation and of mTORC1 activity reduces the capability of cells to spread and migrate. These data suggest that cytoskeletal remodeling requires continuing translation of selected mRNAs and mTORC1 activity, and that mTOR inhibitors can reduce the metastatic spread of cancer cells.

MATERIALS AND METHODS

REAGENTS

Rapamycin was purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma–Aldrich (Milan, Italy).

CELL LINES AND CULTURE

The following human MM cell lines were used: MM98 cells, established from pleural effusion of a sarcomatous mesothelioma [Orengo et al., 1999; Orecchia et al., 2004]; BR95 cells, obtained from pleural effusions of patients with histologically confirmed mesothelioma [Orecchia et al., 2004]; REN cells, a p53 mutant, inflammatory epithelial subtype [Smythe et al., 1994]; MPP89 epithelial-like cells [Orengo et al., 1999]. Mesothelioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Euroclone, Pero, Italy) and 1% antibiotic mixture (Gibco, Invitrogen Life Technologies, S. Giuliano Milanese, Italy).

The immortalized human mesothelial cell line, HMC-htert, [Dickson et al., 2000a] was generously donated by Prof. G. Gaudino (DiSCAFF, University of Piemonte Orientale, Novara, Italy) and was maintained in a medium 199 supplemented with 10% FBS, 200 nM L-glutamine, 3.3 nM EGF, 400 nM hydrocortisone, and 870 nM insulin.

SERUM-FREE CONDITIONED MEDIUM

MM98 cells were seeded in 100 mm plastic dishes with DMEM without serum. The CM was routinely collected, 0.2 μM filtered, and kept at −20°C. Serum-free medium that had not been in contact with the cells was used as the control.

CELL ATTACHMENT ASSAY

Cells were trypsinized, resuspended in medium for 1 h at 37°C, and plated for 45, 60, and 90 min on uncoated or coated six-well plates. Unattached cells were removed by three washes with phosphate-buffered saline solution (PBS) and attached cells were fixed in 3.7% paraformaldehyde, stained with 0.2% crystal violet/20% methanol, and washed with water three times. Stained cells were then solubilized with 1% SDS solution. Once solubilized, 90 μl of solubilized stain was transferred to 96-well plates. Samples were then read in a plate reader (Sirio S, SEAC, Florence, Italy) at 540 nm. The experiments were performed in triplicate.

CELL SPREADING ASSAY

Cells were plated on glass coverslips uncoated or coated with CM. After 45, 60, and 90 min, cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and thereafter actin was stained with fluorescein isothiocyanate-conjugated phalloidin (FITC-phalloidin). Cells were observed with a fluorescence microscope (Axiovert, Zeiss, Oberkochen, Germany). For each experimental point, the surface of 50 cells was measured by means of Scion Image software (NIH Image-Scion Image Software, Version 1.59) and the results were expressed as means ± SD.

FIBRONECTIN DEPLETION

Fibronectin was depleted from CM using CNBr-activated Sepharose 4B (GE Healthcare, Milan, Italy) conjugated to bovine gelatin by following the manufacturer's instructions [Ruoslahti et al., 1982]. The fibronectin depletion was confirmed by Western blotting analysis performed with anti-fibronectin antibody (Sigma–Aldrich).

GEL STAINING

After SDS–PAGE separation, gels were fixed in a solution containing 40% methanol and 10% acetic acid and stained with Colloidal Coomassie brilliant blue G250 (Bio-Rad Laboratories, Hercules, CA) according to Neuhoff et al. [1988].

AUTOMATED PROTEIN SPOT PICKING AND IN-GEL DIGESTION

Gel plugs containing the protein spots were excised using a Proteome Works Spot Cutter (Bio-Rad Laboratories) equipped with a 1-mm diameter needle.

Gel spots were destained (50% methanol, 5% acetic acid), shrunk with 100% acetonitrile (ACN) for 15 min, and dried in a SpeedVac. All excised spots were incubated overnight at 37°C in 20 μl of 25 ng/μl trypsin sequencing grade (Roche, Penzberg, Germany) reconstituted in 50 mM NH_4HCO_3. Peptide extraction was carried out twice using first 50% ACN and 1% trifluoroacetic acid (TFA). All extracts were pooled, and the volume was reduced by SpeedVac.

Each sample was then purified with a reversed-phase ZipTip (Millipore, Billerica, MA) as recommended by the manufacturer.

PROTEIN IDENTIFICATION BY MALDI-TOF MS ANALYSIS

One microliter of tryptic peptides obtained from a single spot was vacuum dried onto a sample target plate with 1 μl of matrix (DHB in 50% ACN/H_2O, 0.1% TFA). Mass spectra were obtained using a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) Voyager-DE Pro BiospectrometryTM Workstation (Applied Biosystems, Framingham, MA). The mass spectrometer was run in a positive ion reflection delayed extraction mode. The spectra were internally calibrated using trypsin auto-digestion peaks at m/z 1153.5741, 2163.0570, and 2273.1600. The data were processed using Data Explorer Software 4.0.0.0 (Applied Biosystems). One missed cleavage per peptide was allowed and a mass tolerance of 50 ppm was used. Oxidations of methionine and carbamidomethylation of cysteine were considered. The proteins were identified searching the NCBI non-redundant protein database on the MASCOT search engine (http://www.matrixscience.com). Results with significant Mascot score ($P < 0.05$) were considered.

IMMUNOBLOTTING

The following antibodies were used: rabbit polyclonal anti-phospho-S6 (Ser 235/236) and anti-rpS6 (Cell Signaling Technology, Celbio SpA, Milan, Italy); rabbit polyclonal anti-4E-BP1 (from N. Sonenberg, McGill University, Montreal, Canada). Cells were lysed with buffer containing 10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.3, 1% Triton X-100, 1% Na-deoxycholate, 1 mM DTT, 5 mM NaF, 2 mM Na_3VO_4, 40 U/ml RNasin® (Promega, Milan, Italy), protease inhibitor cocktail. The crude cell extract was clarified at 4°C at 15,000*g* for 10 min and the amount of protein in the supernatant was quantified by the bicinchoninic acid (BCA) protein assay. The lysate was cleared by centrifugation. Protein content was quantified with BCA as above. Extracts were resolved on SDS–PAGE, transferred to Immobilon-P membranes (Millipore), and probed with appropriate antibodies. Equal amount of proteins was analyzed.

METHIONINE LABELING

Cells were pulsed with 28 μCi per plate of Promix ^{35}S-labeled methionine (GE Healthcare) for 30 min, in the tested conditions. Cells were lysed in 50 μl of RIPA buffer without SDS (10 mM Tris–HCl, pH 7.5, 1% Na-deoxycholate, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail). Aliquots of 10 μl of extracts were TCA-precipitated on glass microfiber filters (Whatman) and counted. Values were normalized on sample protein content, quantified by BCA protein assay. Each sample was performed in triplicate and expressed as mean ± std.

POLYSOMAL PROFILE AND FRACTION ANALYSIS

Polysomal profiles were performed as previously described [Ceci et al., 2003], with some modifications. Cells were pre-treated for 10 min with 100 μg/ml cycloheximide (CHX), scraped in buffer containing 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$, 0.1% Igepal, 100 μg/ml CHX, 40 U/ml RNasin®, protease inhibitor cocktail. Whole cell extracts were clarified at 4°C for 10 min at 15,000*g*. The equivalent of 10 absorbance units at 254 nm were layered on a 15–50% sucrose gradient in 50 mM Tris–acetate, pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM DTT and centrifuged at 4°C in a SW41Ti Beckman rotor for 3.5 h at 39,000 rpm. Absorbance at 254 nm was recorded by BioLogic LP Software (Bio-Rad Laboratories).

MATRIGEL INVASION ASSAY

In vitro invasion assays were performed on a 24-well invasion chamber coated with Matrigel (Becton Dickinson, Buccinasco, Italy). Cells were trypsinized, suspended in DMEM, and plated in the invasion chamber (1×10^5 cells per filter) with or without 50 nM rapamycin. After 6 or 24 h, the cells remaining in the upper chamber were removed by scraping, whereas the cells that invaded through Matrigel and attached to the lower surface of the insert were stained by using 0.5% (w/v) crystal violet solution as described by some authors [Adiseshaiah et al., 2007; Kumano et al., 2008]. Crystal violet was eluted from cells with 33% acetic acid, and absorption of the supernatant from each sample was measured at 540 nm in a plate reader.

RESULTS

FIBRONECTIN-DEPENDENT ADHESION AND SPREADING OF MESOTHELIOMA CELLS

We decided to analyze whether sarcomatous and epithelial mesothelioma cells had different capacities to promote for their capability to promote adhesion to matrix, spreading, and migration. We collected serum-free conditioned media of a sarcomatous cell line, MM98, and of an epithelial cell line, BR95, in order to analyze their capability to support adhesion and spreading. The CM was used to coat plastic dishes. Then, homologous and heterologous cells were plated on CM-coated dishes. We found that serum-free CM of the sarcomatous MM98 cell line was able to induce rapid adhesion of both MM98 and BR95 cells (Fig. 1A,B). Attachment to the matrix was followed by spreading, which was detected by measuring the increase in cell surface area. Quantitative analysis of the attachment kinetics of cells showed that pre-coating the substrate with MM98 CM induced a rapid spreading of both cell lines (Fig. 1C,D) that was accompanied by extensive F-actin remodeling (Fig. 1E). These data suggest the existence of factor/s that are released in the MM98 CM and are able to influence cell adhesion and spreading. The CM of BR95 cells supported attachment, but not spreading (Supplementary Fig. 1).

We identified factors secreted by MM98 and BR95. We collected 300 ml of serum-free CM, both from MM98 and BR95 cells. The collected media were ultracentrifuged and the resulting pellets were separated by SDS–PAGE (Fig. 2A). The visible bands were excised, trypsin digested, and analyzed by MALDI-TOF (Voyager DE-Pro, Applied Biosystems). This latter technique allowed us to identify several proteins (Table I), differentially expressed in the two cell lines. Among them, MM98 uniquely secreted large amounts of

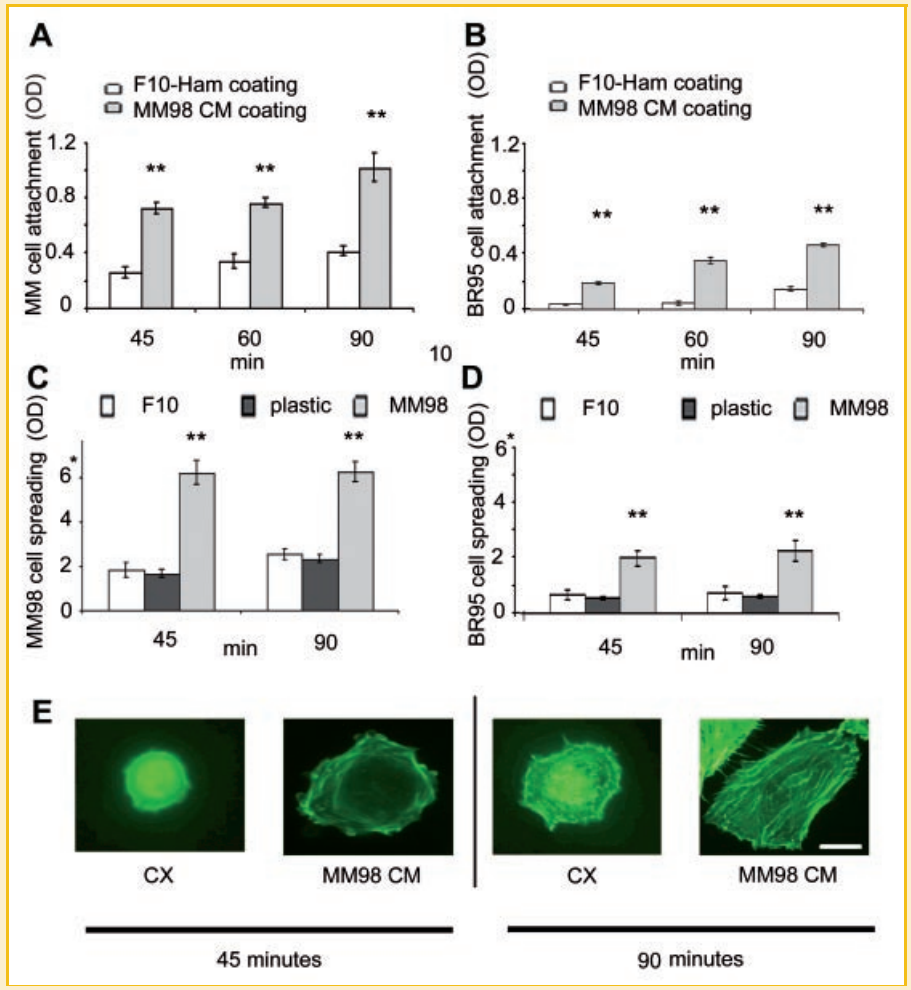

Fig. 1. MM98 serum-free conditioned medium (CM) promotes cell attachment and cell spreading in mesothelioma cells. A: MM98 sarcomatous and (B) BR95 epithelioid cells were seeded in uncoated or MM98 CM-coated 96-well plates. After incubations of 45, 60, and 90 min, cells were analyzed for attachment. The number of attached cells was measured by crystal violet staining followed by reading of absorbance at 540 nm (see Materials and Methods Section). C,D: Time course of spreading of MM98 (C) and BR95 (D) cells with uncoated or MM98 CM-coated coverslips. At indicated times, unattached cells were removed by aspiration and the attached cells were stained with FITC-phalloidin. Spreading was measured by Scion Image Software and expressed as optical density. *10,000×. E: Phalloidin stainings of MM98 cells spreading either on uncoated or on CM MM98-coated wells. Scale bar, 10 μm. Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$.

fibronectin (Fig. 2A). Western blot analysis of conditioned media from MM98 cells and BR95 confirmed that only MM98 cells expressed fibronectin (Fig. 2B, top). On the basis of its abundance and of the existing literature, we hypothesized that fibronectin was the component of MM98 CM, responsible for its pro-adhesive and pro-spreading properties. To verify this assumption, we performed a depletion of MM98 CM by an affinity column on gelatin–sepharose, which is able to remove fibronectin from the sample. Western blotting of CM treated with gelatin–sepharose did not reveal any fibronectin, thus indicating that fibronectin was quantitatively depleted (Fig. 2B, bottom). Next, we measured adhesion and spreading of MM98 cells seeded on wells coated with either CM or with fibronectin-depleted medium. The depletion of fibronectin from the MM98 CM resulted in the reduction to control levels of both attachment and spreading (Fig. 2C,D). Hence, our data show that fibronectin secretion by MM98 cells plays an essential role in cell spreading and adhesion.

ROLE OF TRANSLATION IN THE ADHESION OF CELLS TO FIBRONECTIN

We decided to test whether translation affected cell adhesion to matrix and spreading. Previous experiments in other systems had shown that these processes do not require translation [Stenn et al., 1983]. As previously reported for other cellular models, transcriptional blockage by actinomycin D (2 μM) of mesothelioma cells did not result in the inhibition of spreading (Fig. 3A), suggesting that pre-existing mRNAs are sufficient for matrix-induced adhesion and spreading. In contrast, the general protein inhibitor CHX that acts by blocking peptide elongation impaired the spreading of mesothelioma cells (Fig. 3B). The fibronectin-induced cell spreading was completely abolished by exposure to 50 μg/ml CHX for 90 min, which reduces translation by 90% [not shown and Waring, 1990]. The inhibition of spreading occurred also at lower CHX concentrations, with an EC_{50} of 5.1 μg/ml (95% CI: 3.9–6.6). Spreading was also efficiently inhibited by puromycin, another translation blocker,

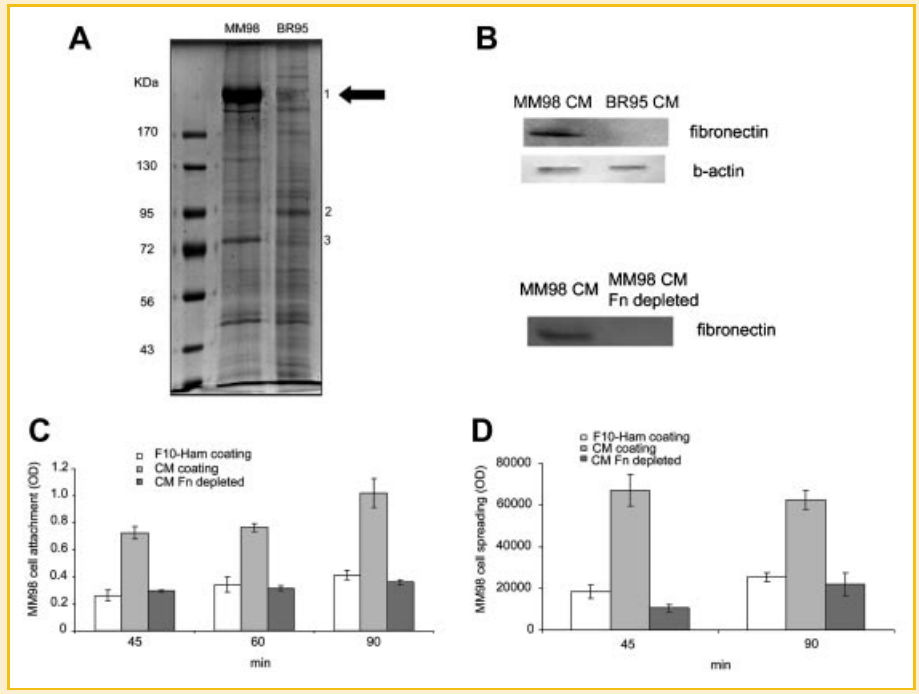

Fig. 2. The fibronectin component of MM98 medium is necessary for inducing cell attachment and spreading. A: SDS–PAGE of serum-free conditioned medium of MM98 or BR95 cells, after centrifugation at 300,000*g* for 2 h. Numbered bands were identified by MS. Arrow indicates fibronectin. B: Top panel: Western blot for fibronectin and actin from conditioned media of MM98 and BR95 cells. Lower panel: Fibronectin depletion by gelatin–sepaharose as determined by Western blot analysis. Equal protein amounts of MM98 conditioned medium and fibronectin-depleted conditioned medium were separated and blotted with an antibody against fibronectin. Effect of Fn-depleted medium on cell attachment (C) and on cell spreading (D) of MM98 cells. Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$.

which acts by inducing premature peptide chain release (data not shown).

Staining of actin filaments revealed marked differences in the organization of actin between control and CHX-treated cells (Fig. 3C). Control cells showed developed stress fibers, whereas CHX-treated cells stained diffusely, showing a scarcely organized cytoskeleton and rare stress fibers. In summary, mesothelioma cells represent the first system, to our knowledge, that requires continuous translation of pre-existing mRNAs in order to efficiently spread.

We have previously shown that the adhesion of non-transformed cells on fibronectin rapidly induces translation in a rapamycin-insensitive way [Gorrini et al., 2005]. We then decided to analyze whether a similar effect was visible in transformed mesothelioma cells plated on their own fibronectin. We plated MM98 cells and control HMC-htert on fibronectin and measured methionine incorporation. Tumor cells had a similar methionine incorporation rate in suspension condition and upon plating on fibronectin, whereas in contrast the control cell line showed a marked increase in translation upon plating on fibronectin (Fig. 4A).

The mTOR pathway regulates cap-dependent translation in cancer cells [Mamane et al., 2006]. We therefore tested whether mTOR affects translation in mesothelioma cells. Rapamycin, which is a pharmacological inhibitor of mTOR, was largely ineffective in reducing methionine incorporation in MM98 cells (Fig. 4B). In spite of this, we found that the mTOR pathway is active in these cells, since the inhibition of mTOR by rapamycin induced dephosphorylation of the targets rpS6 and 4E-BP1 (Fig. 4C). Next, we performed polysomal profiles, allowing to directly estimate the rate of initiation of translation. Rapamycin treatment of cells resulted in modest changes in polysomes/80S ratio (Fig. 4D), indicating that the initiation of translation is largely rapamycin insensitive. Taken together these findings show that in mesothelioma cells the initiation of translation is largely resistant to mTOR inhibition, even though mTOR is activated in these cells.

mTOR-DEPENDENT CELL ADHESION, SPREADING, AND INVASIVENESS

Because of the unusual role suggested by the above data for mTOR, we tested the consequences of mTOR inhibition on cell spreading.

TABLE I. MALDI-TOF Identification of Extracellular Matrix Proteins

Spot no.	Protein ID	Mascot score	Coverage (%)	Queries matched
1	Fibronectin, gi47132555	178	17	36
2	Galectin 3 binding protein, gi5031863	93	16	11
3	Matrix metalloproteinase 2 pre-protein, gi11342666	69	22	12

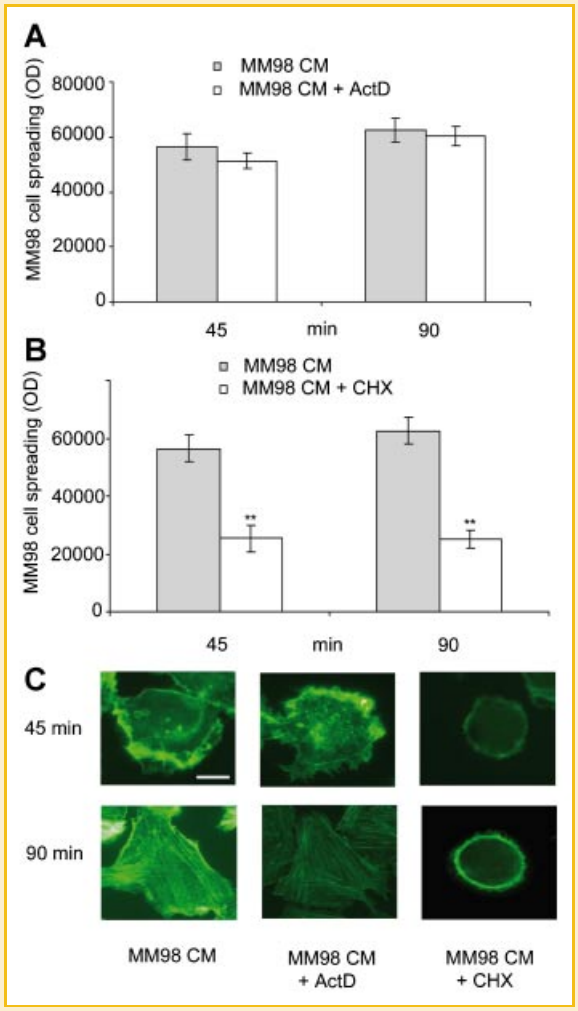

Fig. 3. Effect of actinomycin D (ActD) and of CHX on mesothelioma cell spreading. Cell spreading on conditioned medium is not influenced by the transcription blocker (A), but it is altered by the translation blocker cycloheximide (CHX) (50 μg/ml) (B). C: Effects of CHX and ActD on MM98 cell morphology during spreading. F-actin was stained with FITC-phalloidin. Scale bar, 10 μm. Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$.

Data showed that the inhibition of mTOR by rapamycin is able to inhibit the spreading of MM98 cells on fibronectin (Fig. 5A,B). Similar results were observed on other mesothelioma cell lines (Fig. 6A,B): the epithelial REN cells [Smythe et al., 1994], which have an activated mTOR pathway [Barbone et al., 2008], the epithelial BR95 cells [Orecchia et al., 2004], the MPP89 cells [Orengo et al., 1999], and the immortalized HMC-htert cell line [Dickson et al., 2000]. Also, exposure of these cell lines to CHX yielded data strictly correlated to those of rapamycin, that is, CHX almost completely inhibited cell spreading in mesothelioma cells, while it also affected mesothelial cell spreading (Fig. 6A,B). We also noted that during the spreading phase, the morphology of some cells treated with rapamycin was different from the morphology of cells treated with CHX (Fig. 6A, see, for instance, MM98 and MPP89), suggesting that the two drugs might have different effectors.

We analyzed whether rapamycin could also affect invasion of mesothelioma cells by the Matrigel assay. These experiments showed that MM98 and REN cells treated with rapamycin were consistently inhibited in their capability to penetrate into the Matrigel layer (Fig. 7A,B). This effect was already evident at 6 h after treatment.

DISCUSSION

We have attempted to define differences in the secretion of ECM proteins in sarcomatous mesothelioma versus epithelioid mesothelioma cells. We found that fibronectin, along with other ECM molecules, is highly secreted in a mesothelioma cell line. The presence of fibronectin in pleural effusions of patients affected by MM has been previously reported, but its functional meaning was doubtful [Emri et al., 1997]. Here, we further showed that fibronectin secreted by sarcomatous mesothelioma cells is able to induce also the attachment and spreading of epithelioid mesothelioma cells. During the characterization of this phenomenon, we observed that mesothelioma cancer cells rely on translation in order to efficiently spread on matrix, but their global translation is not affected by mTOR inhibition. Yet, rather surprisingly, this kinase affects mesothelioma cell attachment and spreading onto matrix. Taken together, these data suggest that the assumptions that rapamycin affects cytoskeletal dynamics only indirectly, without affecting mTORC1 complex [Jacinto et al., 2004], and that translation in cancer cells is dependent on mTOR [Mamane et al., 2006] cannot be generalized to MM. We will first briefly discuss the status of adhesion-regulated translation in cancer cells.

One intriguing question is whether MM cells display anchorage-independent translation, and which pathway controls it. Most non-transformed cell types require both growth factors and cell adhesion to a substratum in order to proliferate [Benecke et al., 1978]. This dual requirement is thought to be due to a need for signaling events that originate from growth factor receptors and integrins, respectively [Schlaepfer et al., 1994]. The capability of tumor cells to grow in the absence of substrate attachment is well known and is one of the hallmarks of cancer. However, the capability of translating in the absence of anchorage has never been held as a major determinant of transformation. Indeed, the only report addressing this question dates back to 1981 and was focused on experimentally transformed NIH3T3 cells [Wittelsberger et al., 1981], whereas later reports dealt with longer time points without addressing initiation or signaling [Reiter et al., 1985]. Our study shows that mesothelioma cells maintain high levels of translation independently from substrate attachment. This simple observation suggests that in this way cancer cells can control independently from adhesion major pathways regulating survival and growth, but at the same time may exploit local signals to further shape their expression profile, possibly to their competitive advantage, and in migration-dependent phenomena. The molecular analysis of adhesion-dependent translation induced by fibronectin in non-transformed fibroblasts showed that this process is independent from growth factors, being largely insensitive to mTOR inhibition, but sensitive to PI3Kinase inhibition [Gorrini et al., 2005]. The mesothelioma cell

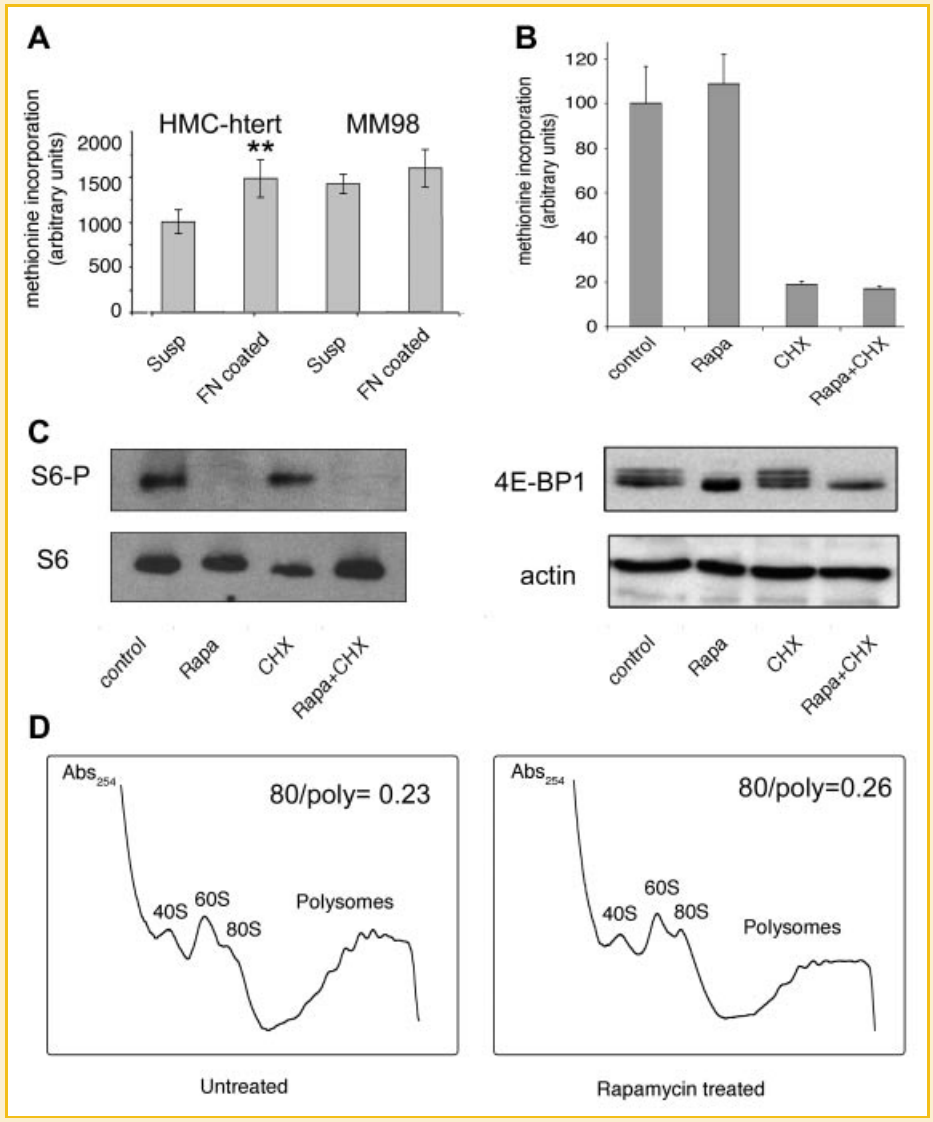

Fig. 4. Translation of MM98 mesothelioma cells is mostly adhesion-independent and insensitive to mTOR inhibition. A: MM98 cells or non-tumoral HMC-htert were serum-starved, kept in suspension or plated on matrix in presence of ^{35}S-labeled methionine, and then assayed for methionine incorporation (see Materials and Methods Section). The experiment was carried out in triplicate. B: The experiment was performed as in (A) and cells were treated as depicted. Rapamycin treatment does not reduce methionine incorporation in MM98 cells (CHX, cycloheximide). Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$. C: MM98 cells were serum-starved and then treated as indicated. Total proteins were resolved by SDS–PAGE and analyzed by Western blot. Rapamycin inhibition reduces phosphorylation of translation-associated factors 4E-BP1 and ribosomal protein S6. D: Polysomal profiles of untreated and rapamycin-treated MM98 cells show no difference in global initiation of translation between control and rapamycin-treated cells. The ratio of 80S to polysomes is shown.

lines that we have analyzed have a constitutively active mTOR pathway, as previously found for other mesothelioma cells [Barbone et al., 2008]. In brief, these data suggest that matrix-independent translation is achieved through the constitutive activation of a growth factor–PI3K–mTOR pathway, whereas the regulation of matrix-dependent translation remains obscure.

Another observation of our study is that inhibition of protein synthesis affects the spreading of mesothelioma cells on their own matrix. Studies performed in the past, employing commonly used cellular models, such as fibroblasts [Neumeier and Reutter, 1985], have invariantly shown that the early phases of attachment and spreading of a cell onto the matrix are largely insensitive to transcriptional inhibition and translational inhibition, indicating that most proteins involved in spreading are pre-existent to cell attachment. In contrast, inhibition of protein synthesis in mesothelioma cells reduced spreading. It must be noted that also insect hemocytes seem to require translation in order to support adhesion and spreading [Nalini and Kim, 2007]. Thus, a straightforward explanation of our findings is that some cell types are unable to accumulate enough proteins to support rapid cytoskeletal rearrangements, thus relying on ongoing translation for spreading. Alternatively, these cells could require the expression of specific peptides involved in the mechanism of adhesion. Therefore, the question to be addressed is which mRNA(s) are

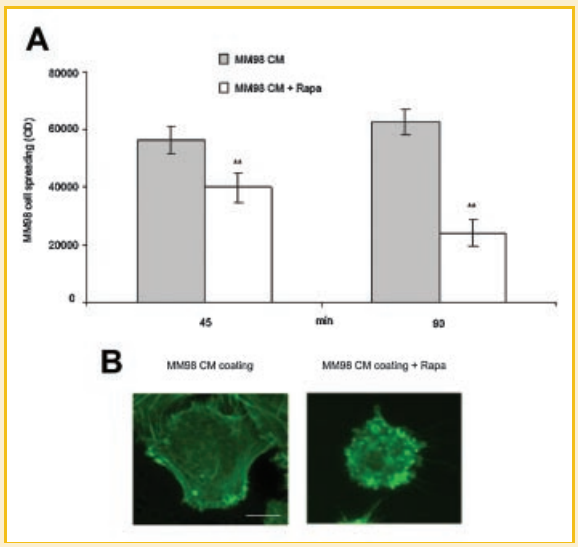

Fig. 5. A: Cell spreading on conditioned medium is altered by rapamycin exposure (50 nM) in MM98 cells. B: Morphology variations of spreading MM98 cells treated with 50 nM rapamycin. Scale bar, 10 μm. Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$.

important in controlling the spreading. Further work is needed to elucidate this point.

Rapamycin is a selective inhibitor of mTOR and therefore it generally affects translational processes. However, previous observations have also linked rapamycin effects to cytoskeleton and cell motility. These results derived from prolonged rapamycin treatments, viz. 48 h exposures in vascular smooth muscle cell [Poon et al., 1996], 20 h pre-incubation for smooth cells [Sakakibara et al., 2005], and 48 h exposure to 1,000 ng/ml for human umbilical vein endothelial cells [Kwon et al., 2005]. Thus, it has been proposed that in mammalian cells a rapamycin-insensitive mTOR complex regulates the actin skeleton. Up to date, two mTOR complexes (mTOR-raptor, TORC1 and mTOR-rictor, TORC2) have been identified [Kim et al., 2003; Jacinto et al., 2004; Sarbassov et al., 2004]. TORC1 regulates phosphorylation of S6K1 and 4E-BP1, while TORC2 phosphorylates Akt [Sarbassov et al., 2005]. TORC1 is rapamycin sensitive, and controls translation, ribosome biogenesis, and other growth-related events. TORC2 is rapamycin-insensitive and regulates polarization of the actin skeleton [Jacinto et al., 2004; Sarbassov et al., 2004]. Here we show for the first time that short-term (60–90 min exposure) inhibition of mTOR by rapamycin exposure decreases cell spreading, cell motility, and invasion.

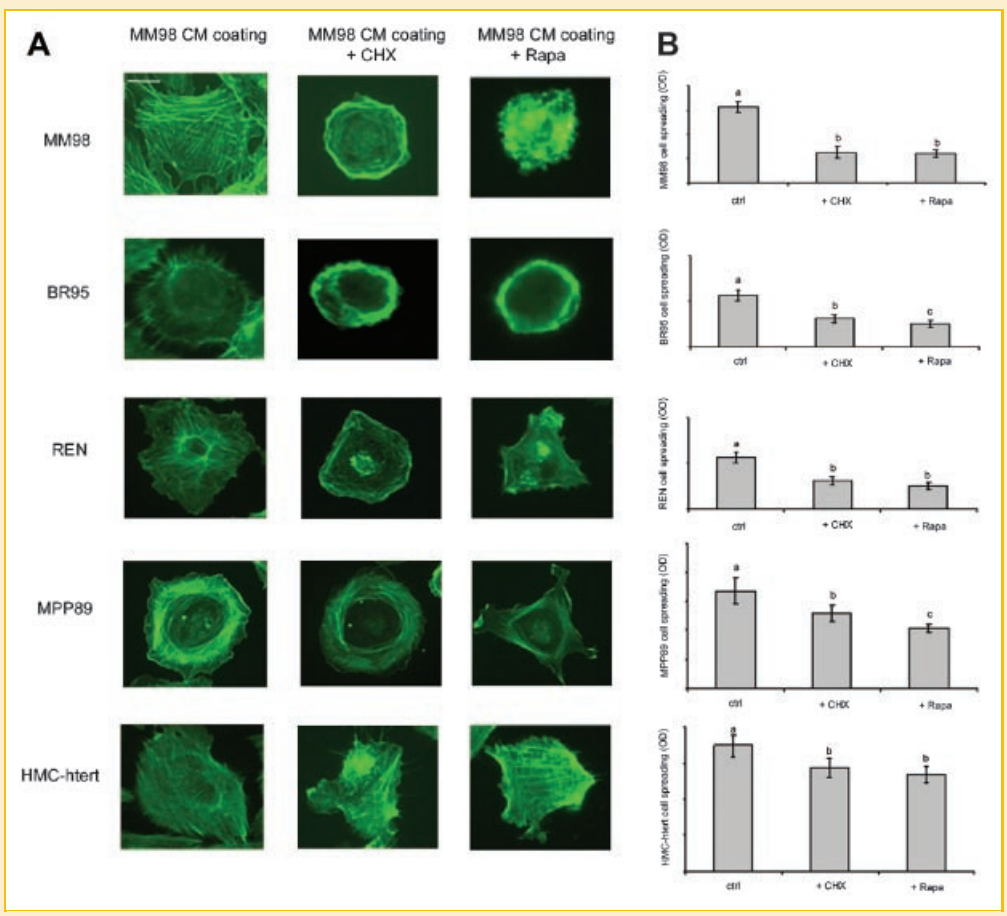

Fig. 6. Effects of rapamycin (50 nM) and CHX (50 μg/ml) on the spreading of four different mesothelioma cell lines and a human mesothelial cell line (HMC-htert). A: FITC-phalloidin staining shows differences in cytoskeletal alterations induced by the two drugs. B: Measurement of cell spreading by image analysis of FITC-phalloidin stained cells, showing various degrees of spreading inhibition in treated cells. Scale bar, 10 μm. Different letters on bars indicate significant differences between groups ($P < 0.01$) according to the Tukey's test.

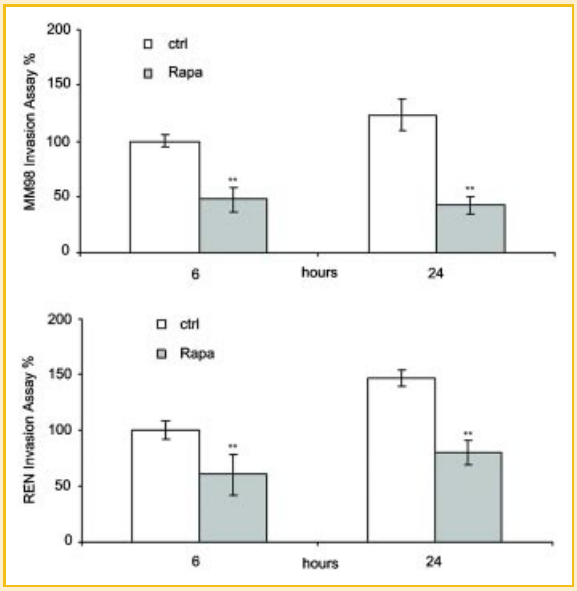

Fig. 7. Rapamycin inhibits the invasiveness of mesothelioma cells. Assay on Matrigel-coated-membrane transwell was carried out on MM98 (A) and REN (B) cells for 6 and 24 h, in the presence or absence of rapamycin. Statistical significance was assessed by the Student's *t*-test. $^{**}P < 0.01$.

In summary, mesothelioma cells seem to possess a specific pattern of control of adhesion to matrix, involving the requirement of fibronectin-induced, mTOR-independent translation, and a rapamycin-sensitive, mTORC1-dependent mechanism of actin skeleton rearrangement (Fig. 8). Such a peculiar involvement of the mTOR pathway in mesothelioma cell adhesion addresses the problem of the possible use of rapamycin-derived chemotherapeutic drugs [Wendel et al., 2006] in the clinical treatment of MM. The scarce anti-proliferative effect of this drug on mesothelioma cells [Barbone et al., 2008] could reflect the occurrence of rapamycin-independent translation in these cells, pointed out by our data, suggesting the unsuitability of the drug for a strategy aimed at tumor growth control. However, the rapamycin-sensitive adhesion and spreading of mesothelioma cells, and the ability of rapamycin to inhibit in vitro cell invasiveness, indicate a possible alternative strategic role of rapamycin derivatives in mesothelioma therapies.

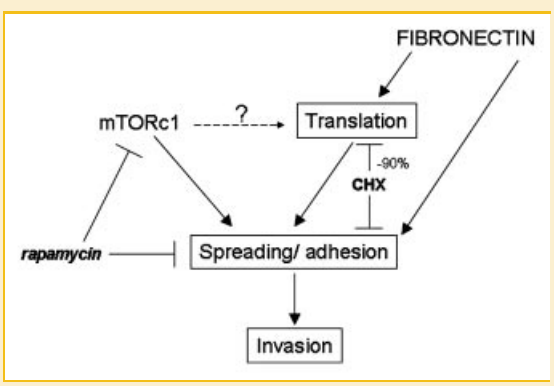

Fig. 8. Schematic diagram of the effects of translation and mTORC1 activity on mesothelioma cell spreading.

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