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Activation of the MAP Kinase Pathway Induces Apoptosis in the Merkel Cell Carcinoma Cell Line UISO

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Merkel cell carcinoma (MCC) is a rare but highly aggressive tumor of the skin. Recently, we have shown that MCC cells *in situ* are characterized by a complete absence of mitogen-activated protein kinase (MAPK) pathway signaling, which is preserved in the MCC cell line UISO. Here we present data suggesting that silencing of the MAPK pathway is essential for the survival of MCC cells. Activation of the MAPK pathway could be achieved by inducing a regulatable form of the c-Raf-1 kinase domain in UISO cells. Consequently, MAPK signaling led to morphological changes, loss of actin stress fibers, and induction of apoptosis, which could be prevented by the MAP kinase kinase-specific inhibitor U0126. Hence, despite the fact that activation of the MAPK pathway contributes to oncogenesis in many cancers, it seems to be a negative selection factor for MCC cells. Since ERK phosphorylation was also inducible by the Raf-activating pharmacological agent ZM336372, these results provide new perspectives for potential therapeutics for this highly aggressive tumor.

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INTRODUCTION

Merkel cell carcinoma (MCC) is a rare form of skin cancer of neuroendocrine origin, with an incidence of 0. 23 per 100,000 people in the Caucasian population of the United States of America (Poulsen, 2004). The tumors are commonly located in body areas that are chronically exposed to the sun, and 95% of the patients with MCC are more than 50 years old (Miller and Rabkin, 1999). Notably, the genetic and molecular mechanisms involved in the development and progression of MCC are not well defined.

Recently, we have reported the virtually complete inactivity of the mitogen-activated protein kinase (MAPK) pathway in MCC as assessed by immunohistological analysis of 49 MCC tumors for expression and phosphorylation of ERK (Houben *et al.*, 2006). This is in striking contrast to the majority of cancers, but is shared by some other neuroendocrine tumors (Kunnimalaiyaan and Chen, 2006a). The MAPK pathway is a major regulator of cell growth, differentiation, and survival and is activated in response to a variety of extracellular stimuli (Naumann *et al.*, 1997). Upon growth factor stimulation, the small G-protein Ras is activated, which leads to the consecutive activation of the three kinases Raf, MEK (MAP kinase kinase), and ERK (p44/p42 MAP kinases).

The importance of the Ras/Raf/MEK/ERK-signaling pathway for carcinogenesis is reflected by the observations that *Ras* genes (*K-ras, H-ras, and N-ras*) are the most frequently mutated oncogenes detected in human cancer (Bos, 1989; Ellis and Clark, 2000) and that B-Raf is activated in several malignancies with the highest frequency found in melanoma (Davies *et al.*, 2002). On the other hand, MAP kinase pathway activation by itself is not sufficient for malignant transformation, as high frequencies of activating B-Raf mutations are also found in benign nevi (Pollock *et al.*, 2003). Moreover, expression of activated B-Raf or Ras in primary cells such as melanocytes leads to cell cycle arrest (Michaloglou *et al.*, 2005). In concordance with the lack of MAP kinase pathway activation in MCC, we and others did not detect any B-Raf or Ras mutations in this tumor entity (Popp *et al.*, 2002; Worda *et al.*, 2005; Houben *et al.*, 2006).

The absence of ERK phosphorylation suggests that MAP kinase pathway activity might be a negative selection factor for MCC cells. Using the MCC cell line UISO as a model system, we demonstrate that activation of the MAP kinase pathway by means of a hormone regulatable fusion protein, consisting of the c-Raf-1 kinase domain and a modified hormone-binding domain of the estrogen receptor (Kerkhoff *et al.*, 1998), induces apoptosis in these cells.

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Abbreviations: MAPK, mitogen-activated protein kinase; MCC, Merkel cell carcinoma; MEK, MAP kinase kinase; OHT, 4-hydroxytamoxifen PBS, phosphate-buffered saline

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RESULTS

Activation of the MAP kinase pathway in MCC cells through a regulatable form of c-Raf-1 kinase

Fusion of the c-Raf-1 kinase domain (BXB) to a modified hormone-binding domain of the estrogen receptor results in a kinase (c-Raf-1-BXB-ER™, here referred to as Raf-ER) whose activity is dependent on the presence of 4-hydroxytamoxifen (OHT), while retaining the same target specificity as Raf (Kerkhoff and Rapp, 1997). To test whether MAPK signaling is inducible in MCC cells by Raf kinase activity, we infected UISO cells with a virus carrying this conditionally active Raf kinase (UISO-Raf-ER) or the empty EYZ vector (UISO vector). Neither unstimulated UISO-Raf-ER cells nor OHT-stimulated UISO-vector cells showed any phosphorylation of ERK (Figure 1). In contrast, a strong phospho-ERK signal was detectable in UISO-Raf-ER cells within 1 hour after addition of OHT, which persisted for 5 days (Figure 1). Notably, the level of ERK protein was equal in UISO-vector and UISO-Raf-ER cells and remained unaltered throughout the whole experiment. It should be further noted that both B-Raf and MEK were expressed in the UISO cells (Figure 2 and data not shown); hence, all the core enzymes necessary for classical MAP kinase pathway signaling were present and functional, as signal transduction could be induced in UISO cells by activating Raf-ER. This indicates that silencing of the pathway in MCC cells actually occurs upstream or at the level of Raf.

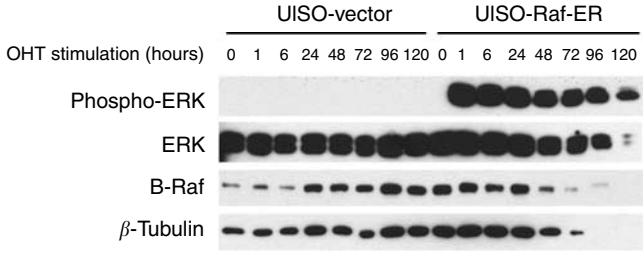

Figure 1. Induction of ERK phosphorylation by OHT stimulation of UISO-Raf-ER cells. UISO-Raf-ER cells expressing c-Raf-1-BXB-ER™ and UISO-vector cells stably transfected with the empty vector were incubated in the presence of 200 nM OHT for the indicated time. Total cell lysates were subjected to Western blot analysis and probed with the indicated antibodies.

Raf activation induces morphological changes and breakdown of actin stress fibers

In the absence of OHT, UISO-Raf-ER cells like UISO vector or the parental UISO cells grow as relatively small, flat cells with rarely appearing extensions of the cell body (Figure 2a). This appearance, however, rapidly changes after activating the kinase; 10 hours following OHT stimulation, first cells start to loose their close attachment to the tissue culture plates, round up and long cytoplasmic extensions become prominent. Twenty-four hours after addition of OHT almost all cells have adopted this phenotype (Figure 2a). The shape of cells depends on so-called actin stress fibers, which are major cytoskeletal structures that are linked to the plasma membrane at focal adhesions (Ridley and Hall, 1992). Fluorescence staining to visualize intracellular filamentous actin structures revealed that the actin stress fibers disappear upon activation of Raf-ER (Figure 2b). Morphology changes

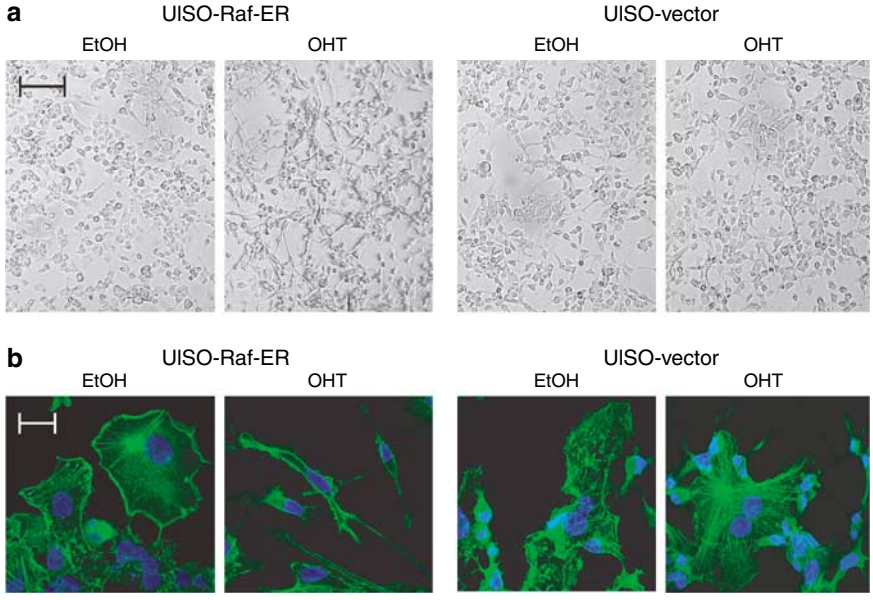

Figure 2. OHT stimulation of UISO-Raf-ER cells induces spindle-shaped morphology and break down of actin stress fibers. (**a**) Light microscopy demonstrating morphological changes induced by Raf activation. UISO-Raf-ER or UISO-vector cells were incubated for 24 hours in the presence of 200 nM OHT or in the presence of the equivalent amount of the solvent EtOH (bar = 100 μm). (**b**) Confocal fluorescence microscopy depicting loss of actin stress fibers upon Raf-ER activation. Forty-eight hours following cultivation of the cells in the presence of OHT or EtOH nuclear staining with DAPI and actin staining with Phalloidin Alex Fluor 488 was performed (bar = 20 μm).

and actin stress fiber breakdown were not observed upon OHT stimulation of UISO-vector cells (Figure 2a and b).

Raf activation induces apoptosis in UISO MCC cells

Upon prolonged incubation of UISO-Raf-ER cells with OHT, an increase in completely detached and shrinked cells became evident, indicating cell death. We therefore analyzed changes in the viability of the UISO-Raf-ER cells over time following addition of OHT. We observed an increase in the percentage of dead cells at 48 hours, reaching a level of 80% at 120 hours (Figure 3a). In contrast, incubation of UISO-vector control cells with OHT or UISO-Raf-ER cells with only

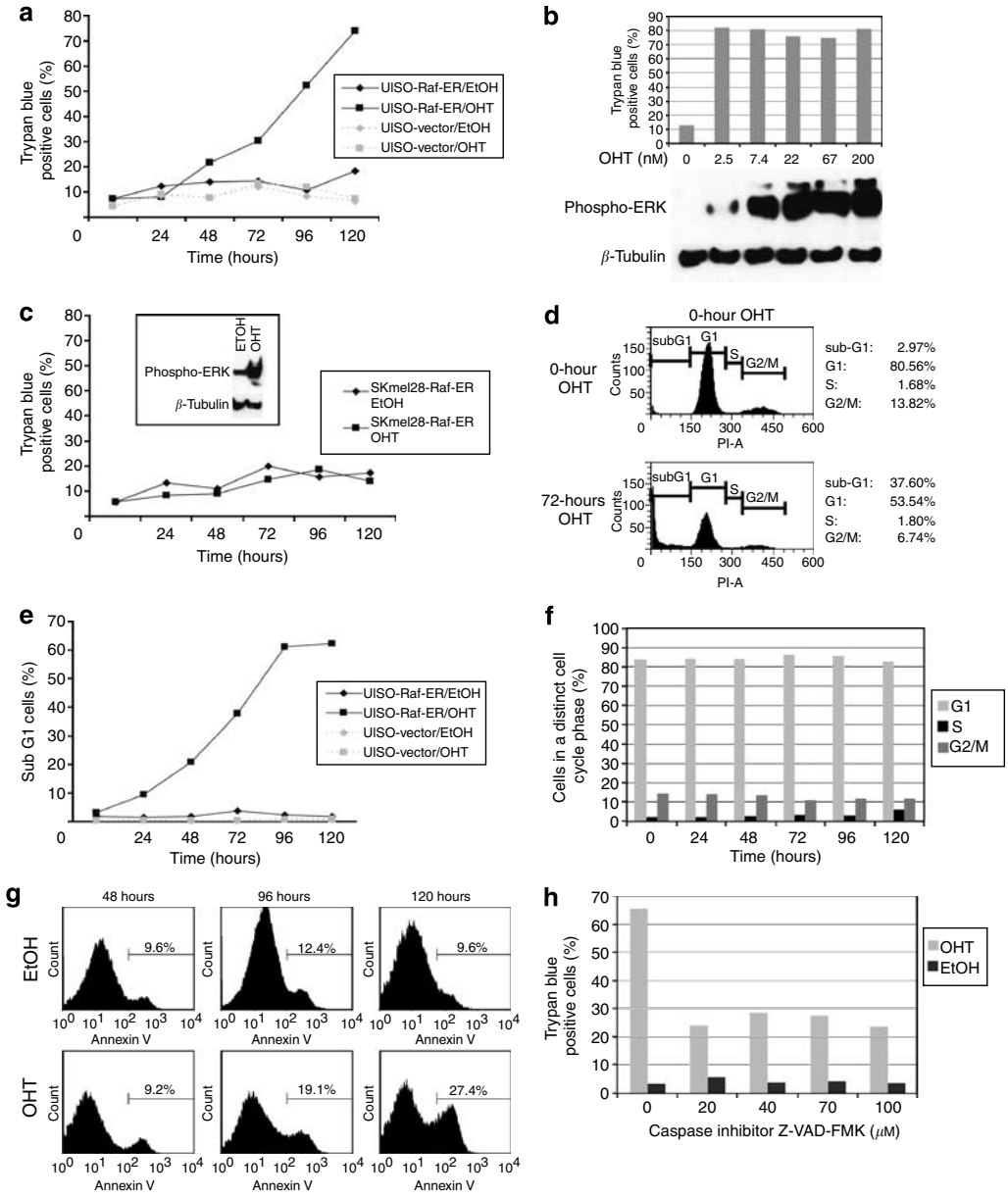

Figure 3. Apoptosis induced by OHT stimulation of UISO-Raf-ER cells. UISO-Raf-ER or UISO-vector control cells were seeded with 2×10^4 cells/well in 96-well plates, followed the next day by addition of either OHT to a final concentration of 200 nM or the solvent ethanol. (**a**) At the indicated time points following OHT stimulation, detached and adherent cells were pooled, pelleted, and resuspended in trypan blue solution. The percentage of cells not excluding trypan blue was plotted against time. (**b**) OHT was added to UISO Raf-ER cells at varying concentrations. ERK phosphorylation by Western blot analysis and trypan blue exclusion was measured following 24 and 120 hours, respectively. (**c**) SKmel28-Raf-ER were incubated either with 200 nM OHT or with the solvent EtOH and trypan blue exclusion was measured in the course of time. In addition, the level of ERK-phosphorylation following 24 hours in the presence of OHT is shown. (**d**) DNA content of UISO-Raf-ER cells was quantitated by propidium iodide staining. Depicted are two examples of flow cytometry histograms of UISO-Raf-ER cells after 0 or 72 hours of OHT stimulation, respectively. (**e**) The percentages of apoptotic cells identified by a sub-G1 DNA content are plotted against time. (**f**) Proportion of living cells in the different cell cycle phases in the course of time following OHT stimulation. (**g**) Annexin V staining in the course of time following OHT stimulation. (**h**) Trypan blue exclusion assay following 120 hours of incubation of UISO-Raf-ER cells either in the presence of OHT or the solvent EtOH in combination with the indicted concentration of the caspase inhibitor Z-VAD-FMK.

the solvent EtOH did not affect their viability. Following extended incubation of UISO-Raf-ER cells with OHT for 10 days, there were no living cells left (data not shown). Titration of OHT demonstrated that even weaker Raf activation associated with only relatively moderate levels of ERK phosphorylation induced cell death in UISO-Raf-ER cells (Figure 3b).

To control the specificity of Raf-activation-induced cell death for UISO cells, we have generated three melanoma cell lines expressing Raf-ER protein. These cells, which display ERK phosphorylation already in the absence of OHT, still showed a strong increase in phospho-ERK upon OHT stimulation (depicted for SKmel28-Raf-ER cells in Figure 3c). In contrast to UISO-Raf-ER cells, however, for all the three melanoma cell lines, there were no signs of increased cell death in OHT-stimulated cells compared with cells treated with the solvent EtOH (shown for SKmel28-Raf-ER cells in Figure 3c). Moreover, two further MCC cell lines MCC13 and MCC26 were transduced with the Raf-ER construct. For both cell lines, we have shown before that they display considerable levels of ERK phosphorylation; therefore, they have not preserved the general *in situ* MCC feature of MAPK pathway inactivity (Houben *et al.*, 2006). Viability of these cells was not affected by MAPK pathway activation through OHT stimulation (data not shown).

It should be noted that for several non-transformed cell lines introduction of active Ras or Raf led to cell cycle arrest, which was at least in some instances mediated by induction of the tumor suppressor protein p21 (Michaloglou *et al.*, 2005). Therefore, we performed a cell cycle analysis to quantitate the distribution of UISO-Raf-ER cells in the different phases following addition of OHT (Figure 3d). Measuring the DNA content by flow cytometry revealed that these slowly cycling UISO-Raf-ER cells contain only a small proportion of cells in S phase. The relative ratio of living cells in G1, S and G2/M phases of the cell cycle remained however relatively constant over a period of 5 days following Raf-ER activation (Figure 3e).

Using the DNA content analysis, a steady increase in the percentage of cells with a sub-G1 DNA content became obvious. This indicates that cell death is accompanied by DNA degradation, a hallmark of apoptosis (Figure 3f). To confirm that cell death induced by Raf activation in UISO-Raf-ER cells indeed occurs through apoptosis, UISO cells were stained with Annexin V, a well-established apoptosis marker. Indeed, cells became Annexin V positive in the course of time following OHT stimulation (Figure 3g). In addition, we applied the pan-caspase inhibitor Z-VAD-FMK. In the presence of Z-VAD-FMK, the extent of cell death induced in UISO-Raf-ER cells by OHT stimulation was substantially decreased indicating the involvement of caspases in mediating apoptosis (Figure 3h).

The MEK inhibitor U0126 blocks ERK activation, morphological changes and cell death induced by activation of Raf-ER

The activation of MEK by Raf and the subsequent activation of ERK by MEK is required to mediate many of the Raf-induced cellular responses, suggesting that ERK is the dominant Raf effector protein (Daum *et al.*,. 1994). However, accumulating evidence indicates that there are additional Raf substrates and that subsets of Raf-regulated events are presumably mediated independently of the MAP kinase cascade (Pearson *et al.*, 2000; Baumann *et al.*, 2000; O'Neill et al., 2004; Gotz *et al.*, 2005). Therefore, by use of the MEK inhibitor U0126, we investigated whether the effects observed in OHT stimulated UISO-Raf-ER cells were indeed MEK-dependent. U0126 inhibited the OHT-induced phosphorylation of ERK, although a relatively high dose of 32 μM U0126 was necessary to completely block ERK phosphorylation (Figure 4a). Increasing the concentrations of this MEK inhibitor, we observed a stepwise-reduced percentage of OHT-stimulated cells displaying the spindle-shaped morphology (not shown) and at 30 μM U0126 no morphological alterations were evident any longer (Figure 4c). The following series of experiments addressed whether cell death induced by activation of Raf-ER was also inhibited by blocking MAP kinase pathway signaling. In the presence of 5 μM U0126, which only partially reduced ERK phosphorylation (Figure 4a), a slight delay in the onset of cell death was observed, whereas in the presence of 30 μM U0126, this effect was much more pronounced (Figure 4b).

MAP kinase pathway activation in UISO cells through a pharmacogical agent

To further confirm that a functional Raf/MEK/ERK cascade is present in UISO MCC cells, we took advantage of the low molecular weight compound ZM336372, which was initially identified as a c-Raf-1 inhibitor *in vitro*, but paradoxically displayed c-Raf-1 activation in cell culture experiments at higher concentrations (Hall-Jackson *et al.*,. 1999). Incubation of UISO cells with ZM336372 at such high concentrations of 100 or 200 μM indeed induced phosphorylation of ERK (Figure 5).

DISCUSSION

Similarly to MCCs *in situ* ERK phosphorylation is not detectable in UISO cells (Houben *et al.*, 2006). Here we demonstrate that MAPK pathway signaling is induced both in UISO-Raf-ER cells by activating the hormone regulatable Raf kinase and in UISO cells by the Raf activating agent ZM336372. Together with the observation that ERK phosphorylation in UISO cells cannot be induced by growth factor stimulation (Houben *et al.*, 2006), these findings suggest that silencing of the MAPK pathway in MCC cells occurs upstream or at the level of Raf.

A candidate for interfering with MAPK signaling upstream of Raf is the retinoid-inducible gene 1 protein, which was shown to block MAP kinase pathway downstream events of all Ras isoforms irrespective of whether Ras was activated by mutation or growth factors (Tsai *et al.*, 2006). This inhibition of Ras action has been suggested to be mediated through both downregulation of Ras expression and through alteration of Ras subcellular distribution. An alternative mechanism of MAP kinase silencing in MCC cells might be mediated by the recently described Prohibitin, which was shown to be required for Ras-induced activation of the Raf-MEK-ERK

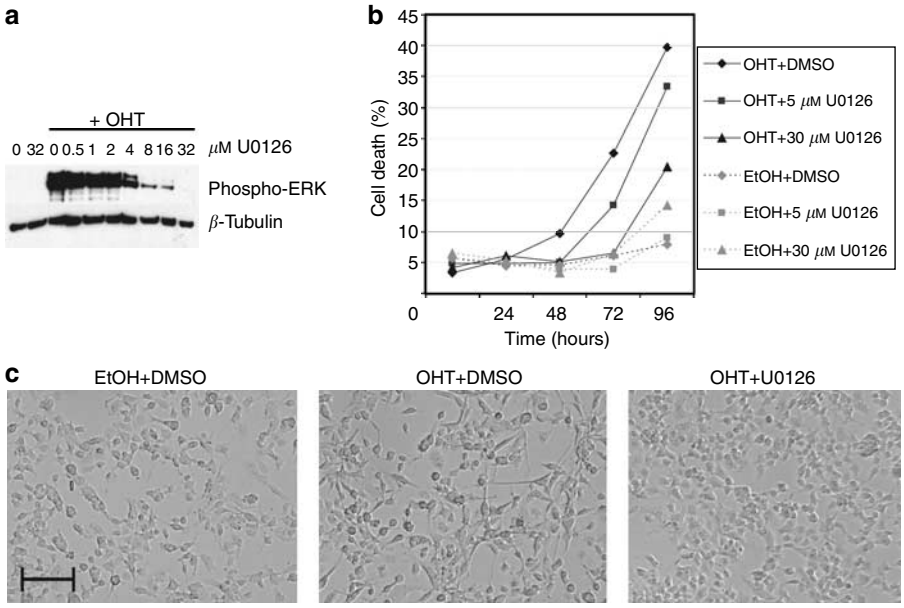

Figure 4. The MEK inhibitor U0126 inhibits ERK phosphorylation, morphological alterations, and cell death in OHT-stimulated UISO-Raf-ER cells. UISO-Raf-ER or UISO-vector control cells were seeded with 0.5×10^5 cells/well in 48-well plates, followed the next day by addition of either OHT to a final concentration of 200 nM or of the solvent EtOH. U0126 or the solvent DMSO was added at the indicated concentrations simultaneously with OHT or EtOH. (**a**) Twenty-four hours after OHT stimulation of UISO-Raf-ER cells in the presence of the indicated concentration of the U0126 total cell lysates were harvested and subjected to Western blot analysis for phospho-ERK and β-tubulin. (**b**) Cell death induced by OHT stimulation of UISO-Raf-ER cells is partially blocked by U0126. The proportion of cells after treatment with OHT or EtOH in the presence or absence of U0126 not excluding trypan blue determined in a Neubauer chamber was plotted against time. (**c**) Light microscopy photographs of UISO-Raf-ER cells following a 24-h incubation in the presence of OHT + DMSO, EtOH + DMSO, and OHT + 30 μM U0126. Bar = 100 μm.

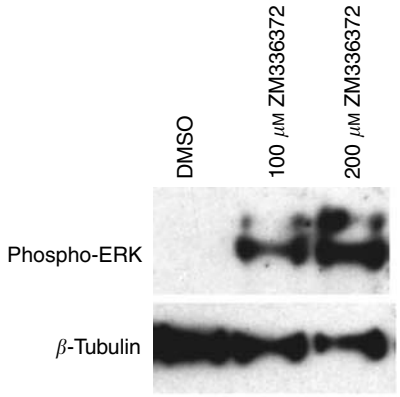

Figure 5. Induction of ERK phosphorylation and morphological alterations in UISO cells by the Raf-activating compound ZM336372. UISO cells were seeded with 0.5×10^5 cells/well in 48-well plates. After 1 day, ZM336372 was added at the indicated concentrations and ERK phosphorylation was detected by Western blot analysis 24 hours later.

module (Rajalingam *et al.*, 2005). Moreover, several phosphorylation as well as dephosphorylation events are involved in Raf activation (Mercer and Pritchard, 2003). Therefore, several modes of silencing the MAPK pathway are conceivable.

Upon activation of Raf-ER by OHT in UISO cells, we observed two major effects, a change in cell morphology and induction of caspase-dependent apoptosis. A spindle-shaped morphology associated with the breakdown of actin stress fibers has been described for Raf transformed NIH3T3 fibroblasts (Kerkhoff and Rapp, 1997). This morphological transformation is regarded as part of the transformation process. The loss of adhesion to the tissue culture plate *in vitro* has been interpreted as mimicking the loss of anchorage to the extracellular matrix and thus the ability to migrate out of the natural tissue *in vivo* (Kerkhoff *et al.*, 2002). This interpretation seems unlikely for an already transformed tumor cell line such as UISO, which has been established from a highly metastatic MCC, and therefore, we propose that the morphological changes rather reflect disturbances in signal transduction pathways regulating cell adhesion and actin stress fiber formation. Indeed, actin stress fibers are maintained by the action of the small G-Protein Rho (Hall, 1998). Raf-ER activation may interfere with signaling downstream of Rho, as sustained Raf/ERK activation was shown to downregulate expression of Rho-associated coiled-coil-containing protein kinase (ROCK) and Rho-kinase, two Rho effectors required for actin stress fiber formation (Sahai *et al.*, 2001).

Recently, induction of apoptosis following Raf-ER activation has also been described for human embryonic kidney 293 cells (Cagnol *et al.*, 2006). Cell death occurred through activation of caspase-8, but was independent of the death receptor FAS and the death receptor adaptor Fas-associated death domain protein. Moreover, the Raf-ER-induced apoptosis did not involve the mitochondrial apoptosis pathway. These and our observations are in contrast to the multiple examples where Raf signaling actually protects cells against

programmed cell death, frequently by inhibiting the mitochondrial pathway of apoptosis (Wang *et al.*, 1996; Zhong *et al.*, 2001; Baccarini, 2002; Gotz *et al.*, 2005).

MEK is the best characterized substrate of Raf, but the existence of additional Raf substrates and alternative Raf effectors has been demonstrated (O'Neill *et al.*, 2004). By the use of the MEK inhibitor U0126, we show that the alterations in cell shape as well as the induction of apoptosis following activation of Raf-ER in UISO cells are both mediated via MEK/ERK signaling. Additional lines of evidence that the morphological changes induced by activated Raf is MAP kinase signaling-dependent stem from experiments using a mutant Raf protein with kinase activity but impaired MEK binding (Pearson *et al.*, 2000). Moreover, consistent with our observation, it was recently shown that Raf-induced apoptosis in human embryonic kidney 293 cells was strictly dependent on MEK signaling (Cagnol *et al.*, 2006).

Raf and MEK kinase inhibitors are currently tested in clinical trials for their effectiveness in the treatment of several cancers, particularly renal cell carcinoma and melanoma (Thompson and Lyons, 2005). However, the use of compounds with an opposite impact has recently been suggested to be useful for the treatment of certain neuroendocrine tumors like medullary thyroid cancer, carcinoid, and pheochromocytoma (Kunnimalaiyaan and Chen, 2006b). A study demonstrating that activated Raf-ER inhibits tumor growth in a xenograft model of human medullary thyroid cancer indicates that this hypothesis is no more exclusively based on *in vitro* data. A possible use of MAP kinase pathway activating agents for the treatment of MCC is highlighted by our findings.

MATERIALS AND METHODS

Cell culture

The MCC cell line UISO, which has been established from a primary MCC of a 46-year-old woman (Ronan *et al.*, 1993), was grown in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. To determine the viability of the cells, detached and adherent cells were pooled, pelleted, and resuspended in trypan blue solution. Subsequently, the percentage of cells excluding trypan blue was determined using a Neubauer chamber. OHT (Sigma, Tautkirchen, Germany), U0126 (Calbiochem, Frankfurt, Germany), Z-VAD-FMK (R&D Systems, Minneapolis, MN), and ZM336372 (Calbiochem) were added to the culture medium; the stock solutions were 200 μM in EtOH, 10 μM in DMSO, 10 mM in DMSO, and 10 mM in DMSO, respectively. The corresponding amount of the appropriate solvent was added to the culture medium of the control cells. The study analyzing exclusively established cell lines was conducted according to the Declaration of Helsinki Principles.

Retroviral transduction

The bicistronic retroviral vector pEYZ has been published previously (Kuss *et al.*, 1999). To obtain pEYZ-c-Raf-1-BXB-ER™, the *Eco*RI fragment of BJ4-BxB-ER™ (Kerkhoff and Rapp, 1997) encoding the c-Raf-1 kinase domain fused to the ligand-binding domain of the estrogen receptor was cloned into the *Eco*RI site of pEYZ. Retroviruses were raised in human embryonic kidney 293 T cells transfected with pHIT60 and pVSVg helper constructs in addition to either pEYZ empty vector or pEYZ-c-Raf-1-BXB-ER™. Two days following transfection, virus supernatants were harvested and filtered through 0.45 μm pore size filters. UISO cells were incubated for 3 days in the presence of virus supernatants, and stably transduced cells were selected in medium containing Zeocin (100 μg/ml). As controls, the melanoma cell lines Skmel28, Mel2a and FM88 and the MCC cell lines MCC13 and MCC26 were infected with the same retroviral vectors and selected as described for UISO cells.

Western blot analysis

For protein analysis, cells cultured under the indicated conditions were lysed on ice in six-well plates using Laemmli buffer (130 mM Tris/Cl pH 6.8, 4% SDS, 20% glycerol, 20 μg/ml bromphenol blue, 4% 2-mercaptoethanol). Testing different methods to harvest cells had revealed that by direct lysis in Laemmli buffer the phospho-ERK signal was preserved best. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Following blocking for 1 hour with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% powdered skim milk, blots were incubated for 2 hours or over night with primary antibody, washed three times with PBS supplemented with 0.05 % Tween 20, and then incubated with the appropriate peroxidase-coupled secondary antibody (DAKO Cytomation, Germany). Following extensive washing, the bands were detected using a chemo luminescence detection kit (Roche Diagnostics, Germany). The primary antibodies used were the monoclonal antibodies, α-Phospho-p44/42 MAP kinase (Thr202/Tyr204) (clone E10; Cell Signaling, Beverly, MA) and α-β-tubulin (Sigma) and the polyclonal antibodies α-RKIP (Upstate, Charlottesville, VA), α-p44/42 MAP kinase, α-Phospho-MEK1/2 (Ser217/221) (both Cell Signaling, Beverly, MA), and α-pan Raf (30 K; a generous gift of Ulf R Rapp (Kerkhoff and Rapp, 1997)).

Fluorescence staining

For fluorescence staining, cells were seeded on sterile coverslips. Following a 48-hour incubation in the presence of OHT or EtOH, cells were washed three times with ice cold PBS and subsequently fixed with 4% paraformaldehyde in PBS for 10 min at 37°C. After three washes with PBS at room temperature, cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min at room temperature, followed again by two washes. Blocking of unspecific binding sites was carried out with 1% BSA in PBS for 1 h at room temperature. Then the cells were incubated for 30 min at 37°C with 1 U of Alexa Fluor 488 tagged phalloidin in 60 μl 1% BSA in PBS. Following two washes, cells were stained with 4′,6 diamidino-2-phenylindole (DAPI) (2 μg/ml) for 15 min at 4°C. Cells were washed twice with PBS and once with distilled water. Cells were covered with Mowiol (Merck, Frankfurt, Germany) and dried for 2 h at room temperature and subsequently over night at 4°C. Then cells were analyzed using a confocal fluorescence microscope (True Confocal Scanner Leica TCS SP II).

Detection of DNA content

Cells in the supernatant as well as cells that still attached to the culture plate were harvested and resuspended in 0.5 ml. PBS supplemented with 1% fetal calf serum. Five milliliter of ice-cold EtOH was added followed by an over night incubation at 4°C. The fixed cells were pelleted and resuspended in 1 ml PBS supplemented

with 1% fetal calf serum, 0.05 mg/ml propidium iodide, and 0.1 mg/ml RNase A. Following a 1-hour incubation at 37°C, analysis of the cellular DNA content was performed on a FACSCanto flow cytometer (Becton & Dickinson, Heidelberg, Germany), and FCS Express software was used to evaluate data.

Annexin V staining

An Annexin V-Cy5.5 conjugate (BD Biosciences, Heidelberg, Germany) was used to identify apoptotic cells by flow cytometry according to the manufacturer's instructions. Analysis was performed on a FACSCanto using the supplied software (BD Bioscience).

Our studies were approved by the institutional review board (Ethik-Kommission der Medizinischen Fakultät der Universität Würzburg; sequential study number 135/06).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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