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Review

JNK and ERK Signaling Pathways in Multistage Mouse Carcinogenesis: Studies in the Inhibition of Signaling Cascades as a Means to Understand their *In Vivo* Biological Role

KOSTAS D. KATSANAKIS[1], CAROLYN OWEN[2] and VASSILIS ZOUMPOURLIS[1]

[1]*Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave, Athens 116 35; and* [2]*Institute of Natural Products, NAICh, P.O. Box 85, Chania 75100, Crete, Greece*

Abstract. *Amplification and mutation of Ha-ras has been shown to correlate with the malignancy of tumors that appear in chemically-induced mouse skin. Cell lines isolated from mouse skin tumors represent the evolutionary stages of tumor development. Due to the high Ha-ras levels the JNK and ERK modules are found elevated, contributing to the enhanced AP-1 activity in the more malignant cells. To examine the role of the transforming Ha-ras in controlling ERK signaling, transfection of an activated Ha-ras allele was tested in a squamous cell carcinoma cell line. The ERK1/2 signaling pathways were blocked pharmacologically by PD98059 MEK inhibitor, which inhibited cell proliferation and anchorage-independent growth of squamous and spindle carcinoma cells. In addition, treatment with PD98059 and introduction of the dominant negative ATF-2 mutant into the spindle carcinoma cells, partially reverted the spindle phenotype to squamous - like. These results suggest that ERK1/2 and ATF-2 play an important role in oncogenicity and in the degree of progression within the mouse skin carcinogenesis system.*

Contents

1. Introduction

Mutations of the ras family of proto-oncogenes are present in 30% of human tumors, contributing to the development of cancer. They are also involved in tumor development in rodents (1). The Ras family of small GTPases are regulated by extracellular signals, such as growth factors, which lead to an increase in GTP-bound Ras (2,3). A major Ras effector is the Raf-1 serine/threonine kinase, which is activated by binding to the GTP-bound state of p21Ras. The activation of the ERK family of MAPKs, *via* Raf and MEK is the best characterized example of Ras-dependent signaling and this activation is clearly required for the transformation of rodent fibroblast, because the expression of the dominant-negative version of MEK, blocks the transformation by Ras (4). ERK1/2 phosphorylate multiple substrates, including transcriptional activators inducing or suppressing transcriptional activation of physiological target genes (5). Some of the targets of ERK1/2 are transactivators that affect the expression and the post-translational modifications of the AP-1 factors (6). Furthermore, ras activates the JNK family of kinases and the phosphatidyl-inositol 3-Kinase pathway through different mechanisms (6, 7). Therefore multiple mechanisms account for the effects of Ras in the regulation of multiple physiological processes, through signal transduction pathways, leading to changes in AP-1 activity.

Mouse multistage carcinogenesis protocols include treatment of the mouse skin at the primary step of initiation with chemical carcinogens which result in mutations of Ha-ras at specific codons that activate its transforming properties (8). Other changes in Ha-ras activity result from its amplification. This is seen in squamous and spindle tumors but not in benign papillomas, suggesting that quantitative changes of Ha-ras also affect neoplastic development, though in different ways than qualitative modifications (9).

The alterations in Ha-ras clearly correlate with an increase in mitogenic signaling, represented by elevated JNK and ERK activities in cells cycling in serum (10, 11). These signals are related to elevated AP-1 activity at late stages of progression. These signals can be reproduced partially if a human allele of Ha-ras is introduced in squamous cell carcinoma cells. Similarly they can be

Correspondence to: Vassilis Zoumpourlis, Insitute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave, Athens 116 35, Greece. Tel: ++ 30 1 7273745, Fax: 30-1-7251827, e-mail: vzub@eie.gr

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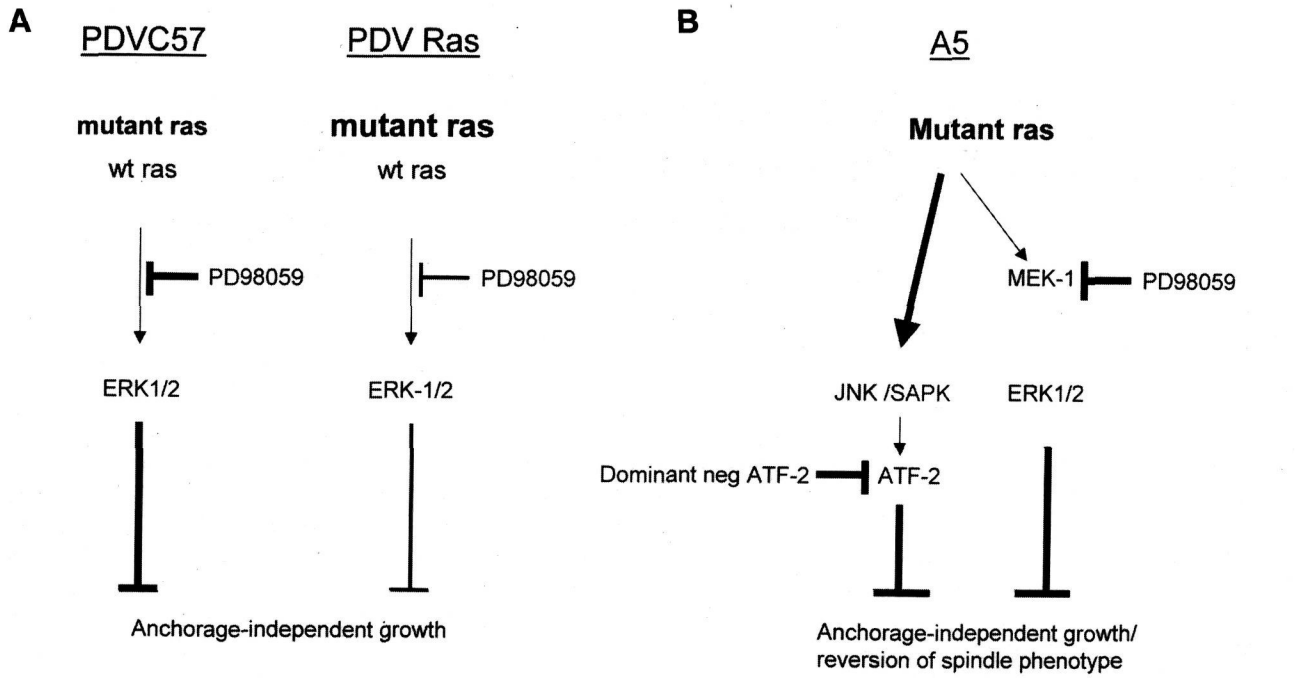

Figure 1. *Suppression of signaling pathways in cells derived from the mouse skin. A) PDVC57 cells have an elevated content of mutant Ha-ras resulting in activation of both ERK-1 and ERK-2 isoforms of MAP kinases. Activation of ERK1/2 in PDVC57 is effectively suppressed by PD98059 during growth in serum. In PDV cells transfected with a transforming allele of human Ha-ras, ERK1/2 activity is elevated and is suppressed by PD98059 to the same extent as in PDVC57, suggesting the direct contribution of Ha-ras to this signaling cascade; B) In A5 spindle carcinoma cells, the expression of the dominant negative ATF-2 is sufficient to induce reversion to a flat fibroblastic morphology. This effect is similar to what was observed by treating A5 with PD98059 where morphological reversion is also evident. The suppression of ERK1/2 activities in A5 is more refractile to inhibition by PD98059 since these cells have sustained ERK1/2 activity when growing instead of cycling conditions.*

inhibited by small synthetic inhibitors of the mitogen – activated protein kinase cascade (11,12).

2. JNK Signaling is Increased in Cells Derived from Mouse Skin Tumors

In experiments with tumor formation in c-*fos* -/- mice there was no progression to malignancy, whereas the control c-*fos* +/+ mice develop tumors, suggesting that AP-1 activity has an oncogenic role and is required for malignant progression *in vivo* (13).

In the cell lines representing the multistage model of mouse skin carcinogenesis the expression of Fos and Jun family members is increased. Fos and Jun protein levels are most clearly elevated in the spindle carcinoma cells and less so in squamous cell carcinoma and some papilloma cell lines (10). This increase affects AP-1-DNA-binding activity and transactivation. It has been well - established that AP-1 transcriptional activity is subject to regulation by signal transduction cascades of the JNK pathway, which is controlled by Ha-*ras* (6).

Investigations of the JNK pathway, using phosphospecific antibodies and solid phase kinase assays, showed that JNK activity also gradually increases during skin tumor progression, thus offering an explanation for the increased AP-1 transactivation. These results highlighted, for the first time, that, in the mouse skin carcinogenesis model cell lines, Ha-ras activation leads to considerable up-regulation of AP-1 activity and also contributes to tumorigenesis, by mechanisms such as the regulation of metalloprotease secretion and activity (14).

3. ERK1/2 Signaling in the Mouse System: the Role of Ha-ras

In fibroblasts transformed by *ras* and Raf-1, ERK1/2 are necessary for transformation and anchorage-independent growth (15, 16). Epithelial cells are less susceptible to transformation by oncogenes than NIH3T3 or Rat-1 cells. *ras* and Raf-1 mutants efficiently transform NIH3T3 cells but only ras mutants induce transformation and growth in soft agar of rat intestinal epithelial cells (RIE) and human mammary epithelial cells (17). Therefore, constitutive activation of Raf/MEK/ERK signaling is not a sufficient cause for transformation of RIE (17). This evidence suggests that Raf/MEK/ERK-independent pathways could be largely responsible for epithelial cell transformation (18).

ERK1/2 phosphorylation showed, like JNK1/2 activity, a progressive increase from papilloma to the squamous and spindle cell lines, implying that both types of kinases are involved in malignant progression of mouse skin tumors (10,

11). Additionally, these results suggest that, in the cells derived from the mouse system, a convergence of multiple signals arise from Ha-*ras* to different downstream effectors.

Balmain *et al* have shown previously that a reduction in the ratio between normal:mutant Ha-*ras* is associated with increased tumorigenicity in squamous cell carcinomas (19). The PDV cell line represents a rare transformant originally isolated by Fusening *et al* (20) after treatment of epidermal cell cultures from newborn mice with the carcinogen DMBA. Karyotypic analysis of PDV cells has indicated the presence of three copies of chromosome 7 (21), on which the H-*ras* gene is located. Analysis at the DNA level has shown that normal and mutated H-ras alleles are expressed at a ratio of 2:1, which corresponds to the relative gene dosage (19). PDV cells, when injected into adult C57B1 syngeneic mice, give rise to squamous carcinoma at only one of the eight sites of injection (20, 21). A cell line derived from this tumor (PDVC57 cells) had a more heterogenous morphology, with an increased number of giant cells and was also more strongly tumorigenic on reinjection into adult syngeneic mice, giving rise to carcinomas at all 5 injection sites (19). Analysis by Southern blotting for the presence of the mutant H-*ras* gene has shown that the ratio of the mutant:normal H-ras genes had changed from 1:2 to 2:1 (19). PDVC57 are 8-fold more invasive and secrete 2-fold more type IV collagenase than PDV. Evidence that PDVC57 are more tumorigenic, chemotactic and invasive than PDV suggests that tumor progression and invasiveness is, at least to some extent, dependent on the normal:mutant H-*ras* ratio (22).

ERK1/2 phosphorylation in PDVC57 is related to the endogenous status of Ha-*ras* oncogene since it can be inhibited by the MEK-1 inhibtor PD98059. To correlate ERK activity directly to the levels of Ha-*ras* in PDVC57 cells, which contain increased levels of mutant Ha-*ras* when compared to PDV, a transfection assay was performed. A transforming allele of human Ha-*ras* was introduced into PDV cells (23; Figure 1A). ERK1/2 phosphorylation in PDV Ras clones was indicative of the Ha-*ras*-dependent activation of ERK1/2 in these cells (11). Therefore in these cells derived from an *in vivo* system, Ha-ras controls ERK activity. This evidence is related to previously published results, where a transforming ras allele introduced into swiss cells induced ERK activation (24).

Given that PDVC57 has more malignant characteristics than PDV, our findings in PDVRas clones imply that ERK1/2 activation may play a role in the degree of agressiveness during the squamous stage of mouse skin carcinogenesis. In order to find out if the ras/MEK/ERK pathway is required for the manifestation of tumorigenic properties of the PDVC57 cells and PDVRas tranfectants, we used a pharmacological approach with a MEK-1 inhibitor, PD98059. The ability of cells to form colonies and grow in an anchorage - independent manner, was inhibited after treatment with PD98059, implying that the proliferation and tumorogenicity of squamous carcinomas of mouse skin are ras/MEK/ERK1/2 pathway-dependent (11; Figure 1A).

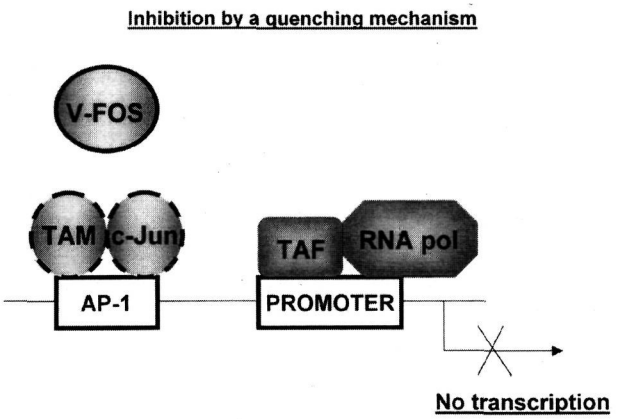

Figure 2. *The activity of TAM-67 in FBR v-fos transformed fibroblasts that is associated with reversion. This is a schematic representation of the possible mechanism that might interfere with transactivation in FBR v-fos transformed fibroblasts by TAM-67 (29). According to Brown et al (29) a quenching mechanism is quite possible in the case of rat embryo cells whereas Angel et al (40) and Ochler et al (41) suggest a squelching mechanism for P9 cells.*

4. Inhibition of Transformation by Dominant Negative Mutants

The Fos and Jun family members are downstream effectors of growth factor and stress signal transduction pathways (6). These transactivators receive multiple signals from the outside of the cell and translate them into altered gene expression. The fos and Jun family members are central to the regulation of many physiological pathways, including those controlling cell proliferation and growth, and behave as either transactivators or transcriptional repressors, depending on the type of extracellular signal and its context.

Inhibition of AP-1 activity by the use of deletion mutants of Fos and Jun proteins, such as the naturally occuring form of fosB, has been documented. The alternatively-spliced form of fosB, DfosB, is a dominant negative inhibitor of AP-1 activity (25, 26). Other studies have used dominant negative-mutants of c-Jun to inhibit transformation by various nuclear and cytoplasmic oncogenes (26 - 29). Lloyd *et al.*, (27) used a v-jun deletion mutant fused to the LexA bacterial moiety, resulting in a chimera that effectively suppresses ras transformation of fibroblasts. The dominant negative N-terminal deletion mutant of c-Jun, TAM-67, can effectively suppresses the effects of transformation by nuclear oncogenes such as SV40, Jun and Fos, and by cytoplasmic oncogenes, such as ras and Raf-1 (27,28). TAM-67 also reverts transformation by FBR v-*fos* oncogene (29). Fibroblasts transformed whith this oncogene are refractile and very invasive (30, 31). Expression of TAM-67 in FBR cells results in the reappearrence of actin stress fibers and suppresses their invasive behavior (29; Figure 2). In squamous cell carcinoma A431 line, the expression of TAM-67 inhibits EGFR-induced cytoskeletal changes (32).

Therefore in various cell contexts, TAM-67 is a very potent dominant negative inhibitor of many of the physiological phenomena associated with the transformed phenotype. The activity of TAM-67 in tissue culture cells might be through a quenching mechanism, where it dimerises with endogenous Jun or Fos proteins resulting in the partial exclusion of those proteins from AP-1 sites, thus preventing normal recruitment of the transcriptional activation machinery components (Figure 2).

In the mouse skin carcinogenesis model system, ATF-2 protein is highly expressed in later stages (10). ATF-2 overexpression by spindle carcinoma cells at a late stage of malignant progression implies that this transactivator might have a positive effect on the AP-1 activity. Therefore it was considered that ATF-2 might be a central element in the maintenance of the spindle phenotype. Transfection of spindle cells with dominant negative ATF-2 mutant (kindly provided by Hans van Dam), lacking the N-terminal phosphorylation sites targeted by SAPK/JNK signaling, caused a reversion of the spindle morpholgy, decreased cell proliferation, and inhibited anchorage-independent growth (33; Figure 1B). Based on these results it would appear that ATF-2 is a central determinant of many of the morphological and genotypic changes occuring during tumor progression in the mouse skin epidermis.

Therefore there is a large body of evidence that dominant negative mutations in AP-1 family members can inhibit transforming signals, initiated by oncogenes acting at diverse signal transduction pathways, in fibroblasts and epithelial cells Conversely, revertant v-fos, transformed fibroblasts are resistant to the action of multiple oncogenes

Discussion

5. The Mouse Multistage Skin Carcinogenesis System and Possible Applications to Therapy

In the cells derived from the mouse model of skin carcinogenesis there is now some evidence that the activity of both the JNK and ERK signaling cascades is increased in the later stages of tumor progression (10, 11). These results suggest an *in vivo* co-operative phenomenon between the two modules, which could increase mitogenic signaling for proliferation. It is possible that the action of both pathways is necessary for tumor progression. Increased JNK and ERK signaling might be the consequence of elevated constitutive Ras activity, resulting from Ha-*ras* mutation and/or amplification. Besides the activation of the JNK and ERK signaling cascades, ectopic ras expression in epithelial cells also results in morphological changes. Transforming ras introduced into human mammary epithelial and MDCK cells has been reported to induce epithelial-mesenchymal transition, resulting in a fibroblastic phenotype (34-36). Signaling by transforming Ras leads to a reduction in Rac activity: Raf/MEK/ERK signaling down-regulates Tiam-1, an exchange factor for Rac, thus down-regulating Rac activity but increasing Rho activity resulting in EMT (37).

Inhibition of JNK and ERK signaling can be achieved through the introduction of dominant negative mutants, or by the use of chemical inhibitors. The use of the MEK-1 inhibitor PD98059 has been documented to reverse v-Ki-ras transformation and to inhibit growth under anchorage independence (38). In the A5 mouse cell line, treatment with PD98059 resulted in partial reversion of the phenotype, from spindle towards a flat fibroblastic phenotype, and also supressed anchorage-independent growth (Figure 1B). Therefore ERK1/2 activities are necessary for anchorage-independent growth, but blocking them is not sufficient to fully reverse the spindle phenotype to an epithelial morphology. This pharmacological inhibition of ERK activation suggests the importance of ERK1/2 signaling in cancer, and indicates possible therapeutic value.

Recent data concerning MEK-1 inhibitors also demonstrated that Raf/MEK/ERK signaling is required for the proliferation of other cancer epithelia. In mouse mammary cancer epithelial cells, ERK1/2 phosphorylation, which is specifically inhibited by PD98059, could be correlated with the EGF-dependent growth and proliferation (39). In mice injected with human or mouse colon carcinoma cells, tumor formation could be abrogated by the MEK-1-specific inhibitor PD184352, which resulted in a reduction in ERK1/2 phosphorylation, tumor volume and invasive index (38).

In conclusion, the inhibition of Raf/MEK/ERK signaling in two different mouse experimental cancer models significantly affected many parameters of tumor development. Since models are thought to represent the complex process of human tumor formation, which is also characterized by multiple changes leading to malignant progression, our results are for special interest: therapeutic strategies that interfere with *ras* signaling might be effective against human epithelial tumors.

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