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AP-1 Transcription Factors and Steroid Hormone Receptors in Multistage Mouse Skin Carcinogenesis

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INTRODUCTION

Tumor development is a multi-step process during which genetic and epigenetic events determine the transition from normal to malignant cellular state. Apart from the fact that tumor cells bear an indefinite proliferative capacity, they also show activation of the signaling pathways that are involved in the transduction of mitogenic stimuli. This activation is either due to overexpression or mutation of signal transduction molecules. Such an example is the activating *ras* mutations, which are present in 30% of the human tumors and result in persistent signal transduction via the MAP kinase pathway, the PI-3 kinase pathway and possibly other downstream effector pathways.

In order to investigate the role of AP-1 transcription factor in carcinogenesis, as well as to identify a potential link between the AP-1, GR and ER factors during this process, we have used a multistage mouse skin carcinogenesis model. This model is ideal for the study of questions related to the timing of oncogene activation and inactivation of the tumor suppressor genes. For the induction of carcinogenesis the most common regimen was followed, which involves administration on the mouse epidermis of a single dose of the polycyclic aromatic hydrocarbon, dimethyl benzanthracene (DMBA), followed by weekly applications of the phorbol ester TPA (12-O-tetradecanoylphorbol-13acetate). Numerous benign papillomas appeared, a proportion of which progressed to malignant carcinomas within 20-40 weeks after the first exposure to the chemical carcinogens. We have selected five cell lines from different stages of mouse skin tumor progression. These include immortalised, non-tumorigenic cell lines (C5N), benign papilloma (P1), squamous carcinoma (B9) cell lines and highly anaplastic, invasive spindle cell lines with highly metastatic properties (A5 and CarB). These cell lines are representa-

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tive of the three distinct stages in mouse carcinogenesis (initiation, promotion and progression).

The initiation and progression of mouse skin carcinogenesis is related to mutations in specific genes, which could endow these genes with transforming properties. A. Balmain carried out a series of studies, which led to the identification of a single gene, the H-*ras* gene (of the *ras* family), as a gene which is consistently mutated after application of DMBA (2). Over 90% of tumors, showed mutations at the middle adenosine residue at codon 61 (CAA) of H-*ras* gene, which resulted in conversion to thymidine (CTA) and in the introduction of an activated missense mutation (2,3). The proof that this gene is implicated in the initiation of mouse skin carcinogenesis came in 1986 by Brown and Balmain, who showed that the retroviruses carrying activated H-*ras* gene when applied to the skin, could replace chemical induction by DMBA *in vivo* (4).

It has already been shown that during tumor progression, genetic changes lead to altered ratio of normal to mutated H-*ras* alleles (5). Such changes involved non-disjunction of chromosome 7 or mitotic recombination leading to homozygosity at the distal part of this chromosome. FISH analysis, which was carried out in A. Balmain's laboratory, revealed that indeed there is an altered ratio of normal/mutant H-*ras* in the mouse skin cell lines. Furthermore, they identified two normal and four mutated alleles in the B9 squamous carcinoma cell line, one normal and two mutated alleles in the spindle A5 cell line and only mutated H-*ras* alleles in the spindle CarB cell line. FISH analysis showed additionally that double-minute chromosomes, in which the H-*ras* gene is located, are present in the A5 cell line.

ONCOGENE AND ONCO-SUPPRESSOR GENE STATUS IN THE MOUSE SKIN SYSTEM

In our laboratory, we have used the same cell lines to investigate whether these genetic changes that lead to altered ratio of normal to mutant H-*ras* at DNA and RNA levels, were reflected to corresponding changes in the protein expression. Indeed, in Table I, lines 1 and 2, it can be seen that there is a different expression of normal and mutant H-Ras proteins between the cell lines, which corresponds to the altered ratio of normal/mutant H-*ras* alleles (6). While C5N immortalised cells expresses only normal H-Ras protein, in papilloma and squamous carcinomas, the balance shifts in favour of the mutant allele of H-*ras* gene. In some spindle carcinoma cell lines, a further increase in expression has been achieved by localised amplification of the mutant H-*ras*, while in other cases, they have lost the normal allele (cell lines A5 and CarB respectively) (Table 1, Ref 6).

Table 1
Qualitative and quantitative changes of AP-1 GR and ER factors during mouse skin carcinogenesis. [1]Method, [2]Molecule, [3]Oligonucleotide, [4]Reporter Plasmid, [5]Western Blot, [6]Retardation Assay, [7]Luciferase Assay

	CELL LINES					
Meth[1]/Mol[2]/Olig[3]/RP[4]	*C50*	*P1*	*B9*	*A5*	*CarB*	*Line No*
WB[5] /N- Ras / - / -	2.0	1.0	2.0	2.0	0	1
WB[5] /M- Ras / - / -	0	2.0	3.0	4.0	2	2
WB / c-jun / - / -	1.0	1.1	2.53	2.81	3.8	3
WB /p-c-jun / - / -	1.0	1.54	2.62	5.4	6.45	4
WB / junB / - / -	1.0	1.16	6.8	2.0	3.0	5
WB / junD / - / -	1.0	0.82	0.95	1.05	0.86	6
WB / c-fos / - / -	1.0	2.4	2.5	5.0	4.5	7
WB / Fra-1 / - / -	1.0	1.1	2.6	2.2	2.9	8
WB / Fra-2 / - / -	1.0	2.5	2.3	2.5	3.0	9
WB / ATF-2 / - / -	1.0	2.2	5.8	4.5	5.1	10
WB / p-ATF-2 / - / -1.0	1.0	2.8	7.9	9.1	12.0	11
WB / JNK1 / - / -	1.0	1.2	2.1	4.25	4.0	12
WB / JNK2 / - /	1.0	1.3	2.0	8.2	.0	13
JNK – assay	1.0	1.1	3.5	3.2	3.2	14
RA[6]/ AP-1/ coll IITRE/ -	1.0	2.1	4.6	7.7	7.6	15
RA/ AP-1/ jun2TRE/ -	1.0	0.9	4.2	5.1	5.6	16
LA[7]/ AP-1/ -/ 5xcollTRE	1.0	2.0	3.78	4.33	4.66	17
LA/ AP-1/ -/ 5xjun2TRE	1.0	1.11	4.31	5.14	6.0	18
WB / ER / - / -	1.0	1.2	1.2	7.0	7.6	19
RA/ ER/ vit ERE/ -	1.0	1.0	3.0	6.0	5,5	20

Mutations in other genes apart from H-*ras* are also found in the mouse skin carcinogenesis model and are also implicated in tumor progression. Mutations in the gene encoding p53 oncosuppressor protein are present in more than half of human cancers. The majority of these mutations are point missense mutations at the DNA binding domains of the protein that inactivate p53 function as a transcrip-

tion factor. In normal cells, p53 levels are increased due to DNA damage and/or other stresses. These increased levels cause dramatic cellular responses, such as cell cycle arrest, senescence and apoptosis. Recent studies have shown that the key element for the control of p53 response is the Mdm2 protein, which exhibits a unique relationship with p53 (7). It has been identified in the mouse carcinogenesis system that loss of heterozygosity (LOH) is present at the p53 locus in one third of the carcinomas. Furthermore, sequencing analysis of the remaining allele of p53 in the B9 and A5 cell lines showed that two mutations are present at codons 236 TGC→TTC and 246 CTT→ CTA (8).

Many members of the cyclin family have been implicated in the etiology of various types of human tumors (9). Specifically, cyclin D1 is a protein which is active in G1 phase and it forms a complex with cyclin dependent kinases 4 and 6 in order to be activated. Their function is negatively regulated by a series of inhibitors including p15, p16, p21 and p27. Overexpression of cyclin D1 leads to shorter G1 period resulting in increased proliferation. In keratinocytes, cyclin D1 expression and the activity of its associated kinases are upregulated in response to oncogenic *ras*. Also in the mouse skin carcinogenesis system cyclin D1 levels are higher in carcinomas than in papillomas and its regulation by *ras* may be transcriptional through the regulation of AP-1 (6,10).

Qualitative changes in the expression of the Rb and p16 oncosuppressor proteins are also present during progression of mouse skin carcinogenesis. The amount of Rb protein is unaffected during cell cycle progression, while the phosphorylation state of Rb is cycle-dependent. pRb is in an uderphosphorylated form in G1, and as the cell progresses into late G1 and early S phase, it becomes hyperphosphorylated and is maintained in G2. Some studies in human squamous tumors have implicated p16 in the immortalisation step of tumor development (11). In contrast, in mouse skin tumors, as well as in human brain tumors and others, a positive correlation has been found between p16/INK4 alterations and the later events in progression (12). In the majority of spindle carcinoma cell lines isolated from mouse skin, i.e. eight out of ten, the INK4 locus was found to be homozygously deleted, while in all of the 20 squamous cell lines studied, the INK4 locus was found to be intact (12).

AP-1 COMPONENTS DURING TUMOR DEVELOPMENT IN MOUSE SKIN

Oncogenic Ras proteins have been shown to regulate AP-1 activity at transcriptional and post-transcriptional levels. Among the *jun* family genes that encode for transcription factors, only *c-jun* displays full transformation potential in cooperation with activated H-*ras* in primary embryo fibroblasts (13). Phosphorylation of N-terminal c-Jun protein residues Ser-63 and Ser-73 by JNK kinases is necessary for transactivation and oncogene cooperation, as shown by *in vitro* transformation assays. Jun B and Jun D can also cooperate with Ras to induce foci, but with lower efficiency when compared to c-Jun (14). Among the Fos protein members it has been shown that Fra-2 plays a crucial role in the transformation induced by H-*ras* gene in CEF, while Fra-1 induces morphological transformation of mouse adenocarcinoma cells and c-Fos expression is involved in benign-to-malignant tumor progression (15-17).

We investigated the role of AP-1 family members in progression of mouse skin cancer. It was detected that there is increased expression of c-Jun in the tumorigenic cell lines, with maximum increase in the spindle cell lines A5 and CarB (line 3). The same pattern of increase was also detected in the expression of the phosphorylated form of c-Jun, as can be seen in Table I line 4. Analysis of the other Jun family members by Western blotting revealed that, whilst the levels of Jun D (lane 6) remained more or less stable in all cell lines, increased Jun B levels were found only in the B9 cell line (lane 5). This result indicates that Jun B overexpression may play a specific role in the formation of the squamous carcinoma phenotype. Western blot analysis was also carried out for c-Fos, Fra-1 and Fra-2 and it was shown that hyperphosphorylated Fra-1 exists in high levels in the cell lines with malignant phenotypes, B9, A5 and CarB (line 8). On the other hand, c-Fos and Fra-2 were found to be elevated in the same cell lines, as well as in the papilloma cell line P1 (lines 7 and 8 respectively) compared to the immortalised C5N cells. ATF-2

protein was also investigated and it was found to be elevated in the papilloma cell line P1, and highly increased in the malignant cell lines, B9, A5 and CarB (line 10). The same was demonstrated also for the phosphorylated form of ATF-2 (line 11).

It was also shown that there are increased amounts of JNK 1 and JNK 2 kinases in the malignant cell lines compared to the immortalised cells (lines 12 and 13). However, JNK 2 is increased to a more modest extent than JNK 1. In order to extend the validity of this result, we analysed the endogenous *in vitro* kinase activity of JNK using GST-c-jun as substrate, and a similar increase was detected as in the Western blot analysis. Since Ras-activated members of the JNK subgroup of MAPKs are the major mediators of c-Jun and ATF-2 aminoterminal phosphorylation (18), the increased levels of JNKs which were detected in the mouse skin, are correlated with qualitative and quantitative changes of c-Jun and ATF-2 proteins (6). From the analysis presented above, it can be seen that altered expression and post-translational modifications of the AP-1 family members are present in the mouse skin cell lines. In order to investigate whether these changes result in the AP-1 DNA binding activity, electromobility shift assays were performed. The DNA-binding activity was found to be slightly increased in the papilloma cell line P1, further elevated in the B9 squamous carcinoma cell line, and peaked in the metastatic cell lines A5 and CarB (Table I line 15, 16). Luciferase activity was also found to be elevated in these cell lines (line 17, 18). Also, it was observed that there is increased c-Jun/ATF-2 binding activity in the malignant cell lines, compared to the immortalised cell line (line 8).

THE IMPLICATION OF STEROID HORMONE RECEPTORS IN MOUSE SKIN CARCINOGENESIS

Glucocorticoid hormones are known to serve a variety of important functions in eukaryotic cells and tissues. Usually, glucocorticoids promote cell differentiation and inhibit proliferation. For example, these hormones are known to inhibit DNA synthesis in normal keratinocytes *in vivo* (19). Studies have demonstrated that glucocorticoids prevent phorbol-ester-induced inflammation and skin hyperplasia (19, 20). Glucocorticoids are very potent inhibitors of skin tumor formation, especially during tumor promotion.

The cellular response to glucocorticoids is mediated through a highly specific cytoplasmic glucocorticoid receptor (GR). Upon activation by ligand binding, GR forms a homodimer and migrates to the nucleus of the cell, where it binds to specific DNA sequences called glucocorticoid-response elements (GREs) (21). The vast majority of GREs have a unique structure; they are palidromic sequences of 15 bp that have been shown to bind a GR dimer and to work as typical enhancers of transcription (20).

The expression of estrogen receptors has been examined. As it can be seen in Table I line 19, Western blot analysis indicated that there is overexpression ER in the spindle cell lines A5 and CarB, compared to the immortalised cell line C5N. This increase indicates that these receptors are activated in the malignant cell lines with metastatic properties. Furthermore, after DNA-binding analysis by electromobility shift assay, it was detected that there is increased DNA-binding on the specific ERE sequences in the same cell line (lanes 20), (Zoumpourlis *et al.*, manuscript in preparation).

CROSS-TALK BETWEEN AP-1 AND STEROID HORMONE RECEPTORS

In contrast to the unique mechanism of gene upregulation by glucocorticoids, through GRE elements, several different mechanisms for gene repression by GR have been described. These mechanisms include competition of GR with other transcription factors for the DNA-binding sites or with common mediators for the transcriptional-initiation complex (21,23-26). This is also evidence of negative cross-talk on the protein-protein level between GR and such nuclear transcriptional factors as AP-1 and inflammation responsive factor NFκB (21,24,27). Thus, genes whose activity is controlled by AP-1 or NFκB may be downregulated by glucocorticoids through inhibition of these factors. The genes that are inhibited by glucocorticoids include prolactin, collagenase type I, stromelysin, GR itself, and some other genes (reviewed in 28). Ultimately, the activation of GR by glucocorticoid hormones

results in activation or repression of the network of glucocorticoid-responsive genes and produces a specific cellular response.

CONCLUSIONS

After the investigation of c-Jun, Fra-1, Fra-2, ATF-2, GR and ER molecules, in this mouse skin model, it was concluded that these proteins play a role in the progression of carcinogenesis, since they were detected at high amounts in the cell lines with malignant phenotypes. The quantitative and qualitative changes in the AP-1 components is probably due to the increased amounts of Ras protein, which is overexpressed in these cells (6). These results are in agreement with previous studies, which note that Ras-mediated transformation activates AP-1 transcription (22). Furthermore, we confirm that ATF-2, together with c-Jun and Fra proteins, appear to be the major components of AP-1 complex in cycling keratinocytes and they are further activated in transformed cells. Additionally we showed for the first time, that dominant negative ATF-2 inverts the spindle carcinoma phenotype to squamous-like. These results indicate the implication of ATF-2 transcription factor at late stages of mouse skin carcinogenesis (Zoumpourlis *et al.*, manuscript in preparation)

Studies of skin carcinogenesis have shown that different target genes exert their functions at different stages of carcinogenesis. The human equivalent of these genes may be useful for the prediction of individual cancer risk, and for the development of approaches to cancer prevention and therapy. The analysis of AP-1 oncogenic components and steroid hormone receptors cross-talking partners in the multistage carcinogenesis system, provide us the tools for the study of the role of hormone agonists and antagonists in cancer therapy.

REFERENCES

1. Quintanilla M., Haddow S., Jonas D., Jaffe D., Bowden G.T., Balmain A.: Comparison of ras activation during epidermal carcinogenesis in vitro and in vivo. Carcinogenesis *12*: 1875-1881 (1991)
2. Balmain A., Pragnell I.B.: Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. *Nature 303*: 72-74 (1983)
3. Quintanilla M., Brown K., Ramsden M., Balmain A.: Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature 322*: 78-80 (1986)
4. Brown K., Quintanilla M., Ramsden M., Kerr I.B., Young S., Balmain A.: v-ras genes from Harvey and BALB murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. *Cell 46*: 447-456 (1986)
5. Bremner R., Balmain A.: Genetic changes in skin tumor progression: correlation between presence of a mutant ras gene and loss of heterozygosity on mouse chromosome 7. *Cell* 1990 *61*: 407-417 (1990)
6. Zoumpourlis V., Papassava P., Linardopoulos S., Gillespie D., Balmain A., Pintzas A.: High levels of phosphorylated c-Jun, Fra-1, Fra-2 and ATF-2 proteins correlate with malignant phenotypes in the multistage mouse skin carcinogenesis model. *Oncogene 19*: 4011-4021 (2000)
7. Thut C.J., Goodrich J.A., Tjian R.: Repression of p53-mediated transcription by MDM2: a dual mechanism. *Genes Dev. 11*: 1974-1986 (1997)
8. Burns P.A., Kemp C.J., Gannon J.V., Lane D.P., Bremner R., Balmain A.: Loss of heterozygosity and mutational alterations of the p53 gene in skin tumours of interspecific hybrid mice. *Oncogene 6*: 2363-2369 (1991)
9. Bartkova J., Jucas J., Muller H., Lutzhaft D., Strauss M., Bartek J.: Cyclin D1 protein expression and function in human breast cancer. *Int. J. Cancer 57*: 353-361 (1994)
10. Gille H., Downward J.: Multiple ras effector pathways contribute to G(1) cell cycle progression. *J. Biol. Chem. 274*: 22033-22040 (1999)
11. Loughram D., Edintgon K.G., Berry I.J., Clark L.J., Parkinson E.K.: Loss of heterozygosity of chromosome 9p21 is associated with the immortal phenotype of neoplastic human head and neck keratinocytes. *Cancer Res. 54*: 5045-5049 (1994)
12. Linardopoulos S., Street A.J., Quell D.E., Parry D., Peters G., Sherr C.J., Balmain A.: Deletion and altered regulation of $p16^{INK4a}$ and $p16^{INK4b}$ in undifferentiated mouse skin tumours. *Cancer Res. 55*: 5168-5172 (1995)
13. Schutte J., Viallet J., Nau M., Segal S., Fedorko J., Minna J.: jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-jun. *Cell 59*: 987-997 (1989)
14. Vandel L., Montreau N., Vial E., Pfarr C.M., Binetruy B., Castellazzi M.: Stepwise transformation of rat embryo fibroblasts: c-Jun, JunB, or JunD can cooperate with Ras for focus formation, but a c-Jun-containing heterodimer is required for immortalization. *Mol. Cell Biol. 16*: 1881-1888 (1996)
15. Suzuki T., Murakami M., Onai N., Fukuda E., Hashimoto Y., Sonobe M.H., Kameda T., Ichinose M., Miki K., Iba H.: Analysis of AP-1 function in cellular transformation pathways. *J. Virol. 68*: 3527-3535 (1994)
16. Kustikova O., Kramerof D., Grigorian M., Berezin V., Bock E., Lukanidin E., Tulchinsky E.: Fra-1 induces morphological transformation and increases in vitro invasiveness and motility of the epitheloid adenocarcinoma cells. *Mol. Cell Biol. 18*: 7095-70105 (1998)
17. Greenhalgh D.A., Wang X.J., Eckhardt J.N., Roop D.R.: 12-0-tetradecanoyl-phorbol-13-acetate promotion of transgenic mice expression epidermal-targeted v-fos induces ras HA-activated papillomas and carcinomas without p53 mutation: association of v-fos expression with promotion and tumor autonomy. *Cell Growth Diff. 6*: 579-586 (1995)

18. Campbell S.L., KhosraviFar R., Rossman K.L., Clark G.J., Dez C.J.: *Oncogene 17*: 1395-1413 (1998)
19. Schwarz J.A., Viaje A., Slaga T.J.: Fluocinolone acetonide: a potent inhibitor of mouse skin tumor promotion and epidermal DNA synthesis. *Chem. Biol. Interact. 17*: 331-347 (1994)
20. Slaga T.J.: Can tumour promotion be effectively inhibited? *IARC Sci Publ* 56, 497-506, 1984. 21. Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. *Cell 83*: 851-857 (1995)
22. Wasylyk C., Imler J.L., Wasylyk B.: Transforming but not immortalising oncogenes activate the transcription factor PEA1. *EMBO J. 7*: 2475-2483 (1988)
23. Diamond M.I., Miner J.N., Yoshinaga S.K., Yamamoto K.R.: Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science 249*: 1266-1272 (1990)
24. Gottlicher M., Heck S., Herrlich P.: Transcriptional crosstalk, the second mode of steroid hormone receptor action. *J. Mol. Med. 76*: 480-9, 1998.
25. Heck S., Kullmann M., Gast A., Ponta H., Rahmsdorf H.J., Herrlich P., Cato A.C.: A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J. 13*: 4087-4095 (1994)
26. Kamei Y., Xu L., Heinzel T., Torchia J., Kurokawa R., Gloss B., Lin S.C., Heyman R.A., Rose D.W., Glass C.K., Rosenfeld M.G.A.: CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell 85*: 403-414 (1996)
27. Caelles C., Gonzalez-Sancho J.M., Munoz A.: Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev. 11*: 3351-3364 (1997)
28. Oakley R.H., Cidlowski J.A.: Homologous down regulation of the glucocorticoid receptor: the molecular machinery. *Crit. Rev. Eukaryot. Gene Expr. 3*: 63-88 (1993)