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Normal *ras* genes: Their onco-suppressor and pro-apoptotic functions (Review)

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Abstract. The *ras* family of oncogenes has been extensively studied for its implication in several types of human malignancies. Activation of *ras* genes involves mutations that alter the catalytic activity of the protein enhancing the downstream signals mostly towards cell proliferation and malignant transformation. *Ras* genes are also involved in induction of senescence or apoptosis, suggesting activation of alternative pathways that may be anti-oncogenic. Early experiments showed that transfection of wild-type *ras* in transformed cells reversed the oncogenic phenotype suggesting that wild-type *ras* has onco-suppressive properties. Indeed, expression of wild-type *ras* genes in several human malignancies is associated with good prognosis. In tumors carrying mutant *ras* genes the levels of expression of the wild-type allele never exceeded the mutant counterpart, indicating that the wild-type protein suppresses the effect of the mutant one. Recent development of the Kras2 deficient mice provided the tool to study the role of wild-type *ras* genes in tumorigenesis.

Contents

1. Introduction

The ras family members are GTP binding proteins placed at the top of an administrative cascade of molecules. Their natural role is to relay extracellularly derived signals to a number of pathways controlling cellular proliferation and differentiation. *Ras* genes have been shown as major participants in the development and progression of a series of human tumors (1). Nevertheless, early observations provided evidence indicating that expression of wild-type *ras* genes possesses anti-oncogenic properties. Proof that *ras* has onco-suppressive activity came very early when Spandidos and Wilkie demonstrated that expression of the normal H-*ras*1 gene suppresses the transformed phenotype induced by the T24 mutant H-*ras* (2) or the mutant N-*ras* (3) present in tumor cell lines. Recent development of the K*ras*2 deficient animals provided the tool to study the role of wild-type K*ras*2 gene in tumorigenesis (4).

2. Onco-suppressive function of wild-type *ras* genes

Ras proteins are small GTP binding proteins, and were amongst the first molecules identified for their involvement in cell transformation and tumorigenesis. Since then, compelling evidence has accumulated implicating the activation of *ras* genes in various human malignancies (5,6). *Ras* genes contribute to tumorigenesis through accumulation of mutations that render the protein highly active. Thus, wild-type *ras* possesses a weak GTPase activity while its mutant counterparts have several-fold stronger catalytic activity. Mutant *ras* genes have the ability to induce malignant transformation in NIH3T3 cells. Since these cells contain and express the normal *ras* alleles it was believed that mutant *ras* was dominant in determining the transformed phenotype. However, transfected cells often express the exogenous genes at much higher levels than the resident alleles because of integration of multiple copies or due to transcriptional regulation, since transfected gene expression is often driven by strong viral promoters. To address the question whether mutant *ras* genes are dominant over their wild-type counterparts, Spandidos *et al* (3) transfected different ratios of mutant and wild-type *ras* genes, expressed under the same promoter, in rat 208F fibroblast cells. Expression of wild-type *ras* significantly inhibited anchorage-independent growth and colony formation that is strongly induced by the mutant *ras* gene (3). In addition, transfection of wild-type *ras* in cells transformed with the T24 mutant *ras* gene was sufficient to reverse the malignant phenotype. In some cell types the amount of wild-type protein had to be significantly higher than the mutant one in order to reverse the malignant phenotype, as indicated from the experiments in the EJ bladder carcinoma cell line. The experiments utilized wild-type and mutant H-*ras*. In HT-1080 cells transformed with the mutant N-*ras*, expression of wild-type H-*ras* partially reversed the

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phenotype induced by the mutant N-*ras* (2,3). This observation indicated that all the *ras* genes may also play an onco-suppressive role.

K-*ras* has been implicated in various human tumors including head and neck and lung cancers. Methylene chloride-induced lung carcinomas in mice were associated with loss of both wild-type K*ras*2 alleles. Construction of the K*ras*2 deficient mice provided a new tool to study the mechanisms of K-*ras* induced oncogenesis. Mice lacking both K*ras*2 alleles die within 14 days of gestation. Mice lacking one K*ras*2 allele are more predisposed to the development of lung tumors by carcinogens compared to mice containing both alleles. Similarly, in the mouse lung tumor cell line LM2 expression of wild-type K*ras*2 inhibited growth and colony formation, indicating that wild-type K*ras*2, in a similar fashion as wild-type H-*ras* has anti-oncogenic properties (4,7).

The mechanism under which *ras* induces transformation has been extensively studied. The key role of *ras* in several physiological and tumorigenic signals has been analyzed in detail. *Ras* is a binary GDP/GTP switch in the inner surface of the plasma membrane relaying signals from receptors to the cytoplasm. Ras proteins receive farnesyl molecules that anchor them onto the membrane, and signals to activate the Raf kinase and the MAPK pathway via ERK1/ERK2 (8). Activation of the MAPK cascade is vital for cell proliferation and enhanced activation of this pathway contributes to the development of the transformed phenotype. A possible explanation of the mechanism of action for wild-type K*ras*2 comes from the observation that there is an inverse correlation between levels of expression of wild-type K*ras*2 and ERK activation, also correlated with the degree of transformation. Thus, in cells that express high levels of wild-type K*ras*2, ERK activation by EGF is lower than in cells expressing low levels of K*ras*2.

In cell culture experiments it was shown that expression of *ras* genes not only contributes to cell proliferation and transformation but, under certain circumstances, it drives cells into senescence. Expression of oncogenic *ras* can induce transformation only in cooperation with another oncogene such as *myc*, E1A, SV40 large T antigen, or when an onco-suppressor gene like p53 or p16 gets inactivated (9). When mutant *ras* is expressed in non-transformed fibroblasts it results in elevation of p53, p16 or p21 levels and cellular senescence (10). Similarly, expression of Raf kinase, the main signaling target of *ras*, drives fibroblasts into senescence suggesting that activation of the ras/Raf signaling pathway promotes senescence, highlighting another cellular mechanism to prevent proliferation of cells that have acquired mutations in *ras* genes (11). Concomitant inactivation of p53 or p16 suppresses this mechanism. It should, thus be tested whether presence of the wild-type *ras* gene has an effect on the activation of p53, p16 or other genes that promote arrest of the cell cycle in the G1 phase. The mechanism through which expression of wild-type *ras* gene inhibits the action of the mutant type may lie in the activation of certain signaling pathways that promote either senescence or apoptosis, or alternatively in the competition for the same substrates. The exact mechanism remains to be elucidated.

3. Involvement of *ras* genes in apoptosis

Although initially *ras* was identified as an oncogenic factor, its involvement in the regulation of apoptosis, and consequently cell death, has now emerged convincingly. A great number of studies have associated the *ras* family members with suppressive and apoptotic effects. Early experiments in 208 fibroblasts by Wyllie *et al* showed that normal Ha-*ras* promoted apoptosis in a similar manner as c-myc, while T24*ras* inhibited apoptosis and strongly promoted proliferation (12). On the other hand, substantial research work demonstrates that *ras* expression protects cells from apoptosis. These concepts seem initially contradictory, however, they could co-exist taking into account the variety of regulatory elements in the different cell systems as well as the differences in the amplitude and the persistence of the extracellular stimuli that are enforced. We will present the outline of the established pathways involving *ras* and leading to cell death modulation along with some intriguing research reports implicating novel molecules.

Promotion of cell death. Active induction of cell death by *ras* is being mediated mainly by apoptosis although there is a report demonstrating a 'type 2 physiological cell death' (13). The biological systems utilized in these studies include T-cells, fibroblasts or cancer specific cell lines. *Ras*-dependent induction of apoptosis in fibroblasts has been demonstrated in response to a variety of external signals such as the loss of adhesion to the matrix (14,15), treatment with tamoxifen (16) and tumor necrosis factor (17). Also it has been demonstrated that *ras*-mediated production of superoxide anions determined sensitivity to intracellular induction of apoptosis among fibroblasts (18).

Fibroblasts can be manipulated to induce apoptosis through tipping the balance of certain intracellular pathways. Using *ras* mutants, Kauffmann-Zeh *et al* (19) demonstrated that activation of Raf by *ras* promotes apoptosis in fibroblasts containing an inducible c-Myc oncoprotein, whereas activation of PI3K by *ras* promoted cell survival.

The pro-apoptotic pathway affected by the *ras* over-expression was found to be the Raf/MAP kinase (19). Induction of apoptosis was reported to be dependent on functional p53 (15,16,20), a fact that links the *ras*-induced pro-apoptotic events with the cell cycle regulation mechanism. Thus, it was proposed that this mechanism is part of a protective response of cells against excessive activation of the MAP kinase pathway (21).

T lymphocytes employ a precise series of activation signals in order to promote their proper activation and differentiation. The slightest deviation causes induction of apoptosis through the Fas-FasL pathway and/or growth arrest. Overexpression of *ras* in T cell lines upregulates expression of FasL in cooperation with activation signals such as IL-2, whereas dominant negative mutants of *ras* inhibit FasL expression in response to stimulation through the TCR complex (22,23).

Recently a novel *ras* mediated pro-apoptotic pathway was identified by studying the Ha-RasG12V E37G, an effector loop mutant that is unable to utilize the Raf or PI3-kinase pathways (24). Interference experiments indicated strongly that the pro-apoptotic stimulus supplied by both Ki-RasG12V

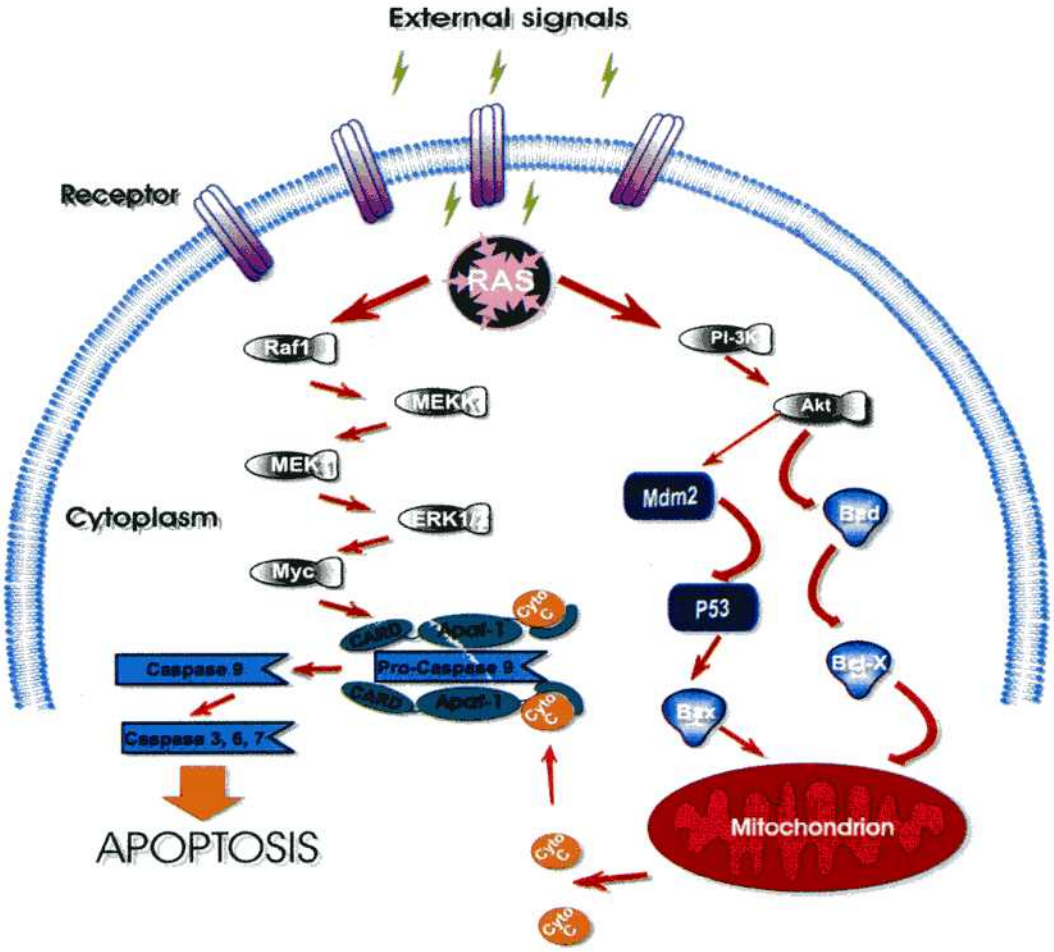

Figure 1. Schematic representation of the major ras-mediated pathways involved in the regulation of apoptosis.

and Ha-RasG12V, E37G required the participation of NORE and MST1. Thus, overexpression of the C-terminal segment of NORE that binds MST1 *in vivo* was sufficient to suppress almost completely the pro-apoptotic action of Ki-RasG12V and Ha-RasG12V, E37G. Similarly, overexpression of the C-terminal non-catalytic segment of MST1, which contains the MST1 autoinhibitory, dimerization, and NORE binding domains, also strongly suppressed the pro-apoptotic action of Ki-RasG12V and Ha-RasG12V, E37G.

Prevention of cell death. Ras mediated signals that inhibit apoptotic cell death has been shown to act through PI3-kinase and AKT pathway (Fig. 1), both leading to a block of cytochrome c release from mitochondrial membranes (25). This is achieved mainly by AKT mediated phosphorylation and thus functional inactivation of BAD which normally is destined to promote cytochrome c release by counteracting the anti-apoptotic function of Bcl-2 and Bcl-XL (26,27). Moreover, AKT was shown to have a cytochrome c independent anti-apoptotic effect through direct inactivation of caspases 9 and 3 most probably by direct phosphorylation (28). A recent report also implicates AKT in the active destruction of p53 (a mediator of apoptosis) through the Mdm2 pathway (29).

A novel *ras*-mediated apoptotic model in which *ras* functions as a sensor of caspase activity to determine whether or not a cell should survive was reported (30). When caspases are mildly activated, the partial cleavage of RasGAP protects cells from apoptosis. When caspase activity reaches levels that allow completion of RasGAP cleavage, the resulting RasGAP fragments turn into potent pro-apoptotic molecules.

The precise role of *ras* in the regulation of apoptosis is still under investigation, and is complicated further by a research report implicating *ras* in 'type 2 physiological cell death' or 'autophagic degeneration' which is a process distinct from the molecular mechanism of apoptosis (13).

4. Genetic evidence supporting the anti-oncogenic properties of *ras* genes in human tumors

Ras genes which function as a molecular switch in a large network of signaling pathways, have been recognized as a major etiological factor for the initiation and the development of human tumors. These alterations are either point mutations occurring in codon 12, 13 or 61 resulting in continuous stimulation of cell proliferation or, alternatively, a 5- to 50-fold amplification of the wild-type gene (1). It has been suggested that mutations at the above codons confer a proliferation advantage in the cells bearing these mutations and thus they are selected within the cell population as compared to other mutations in different sites of the *ras* genes (1). Activated *ras* oncogenes have been detected in a significant proportion of all human cancers but the incidence varies considerably among

Table I. *Ras* mutations in human tumors.

Tumor site	Predominant *ras* isoform	Mutation frequency (%)
Non-small cell lung cancer (adenocarcinoma)	K	33
Colon	K	44
Small intestine	H	31
Pancreas	K	90
Liver	N	30
Myelodysplastic sydrome	N, K	40
Acute myelogenous leukemia	N	30
Skin	H, K, N	46
Ovary	K	48
Cervix	K	20
Endometrium	K	10-40
Thyroid		
Follicular	H, K, N	53
Undifferentiated papillary	H, K, N	60
Papillary	-	-
Head and neck	H, K	30
Bladder	H	10
Breast	K	12
Brain	N, H	13
Kidney	H	10

K, Kirsten; H, Harvey; N, neuroblastoma.

the tumor types (1). It is noteworthy that the mutations are frequently limited to only one of the *ras* family members and the frequency is tissue- and tumor type-dependent (31).

The incidence of *ras* mutations varies greatly among different sites of human tumors (32). A summary presenting the frequencies of *ras* mutations detected in tumors located in various sites is shown in Table I. The highest incidence was found in adenocarcinomas of the pancreas (90%). Mutations in both pancreas and colon cancer have been found in the K-*ras* gene, exclusively. In cancers of the urinary tract and bladder, mutations are primarily in the H-*ras* gene while N-*ras* mutations are dominant in brain tumors and in leukemia. Overall, mutations most frequently occur in K-*ras* and rarely in H-*ras*. Thyroid carcinomas are unique by harboring mutation in all 3 members of *ras* family genes.

All these afore-mentioned mutations in human tumors, both naturally occurring and experimentally induced, support the role of *ras* genes in inducing malignant transformation. However, numerous studies have been carried out in human carcinomas providing evidence for an additional function of the *ras* genes acting as tumor suppressor genes. Studies in human colorectal carcinoma specimens revealed that the levels of the wild-type K*ras*2 mRNA never exceeded the mutant counterpart (33). In human squamous cell carcinomas of the head and neck expression of wild-type H-*ras*1 has been associated with favorable prognosis (34). Analysis of the H- K- and N-*ras* genes for expression, mutation and amplification in both laryngeal and breast tumors revealed significant rates of overexpression in all the members of the family but no point mutation, indicating that the wild-type alleles have been retained (35,36). According to previous studies showing that transgenic rats expressing the wild-type human H-*ras* gene driven by a human H-*ras* truncated promoter element did not develop carcinomas in the mammary gland (37,38) increased expression levels of the wild-type alleles may function rather in favor of reducing the transforming potential than of promoting the malignancy. An additional line of evidence supporting the tumor suppressive function of H-*ras*1 derives from the detection of allelic imbalance at the microsatellite element located within the first intron of the gene in breast cancer (39). Considering that breast tumors do not harbor point mutations in the H-*ras* gene (36), the deleted allele should be the wild-type one.

Activated *ras* is generally believed to have transforming capacity even when the normal wild-type gene is present. However, additional events, such as overexpression or amplification of the mutant allele, or loss of the wild-type allele may need to occur to promote a malignant phenotype (40). Apart from the afore-mentioned allelic imbalance in breast cancer there are a number of studies showing considerable loss of heterozygosity (LOH) at the K-*ras* genetic locus in a variety of human tumors, including lung adenocarcinomas (41,42), prostate (43) and pancreatic cancer (44) as well as in acute lymphoblastic leukemia (45). Unfortunately, there is only a limited number of studies on LOH at the K-*ras* region and on any possible correlation with K-*ras* mutations.

Wild-type *ras* allele has been proposed to inhibit cell proliferation by promoting differentiation (7). It has been shown that ras proteins can induce differentiation of cells such as neurons under certain conditions (46). Similar correlations between the occurrence of mutations in H- or K-*ras* genes and the differentiation of tumors have been observed in human malignancies, such as salivary gland mucoepidermoid carcinomas (47) and gastric cancer (48) supporting this hypothesis.

5. Concluding remarks

Overall, the balance between proliferation and tumorigenesis versus apoptosis and senescence seems to be regulated by a sophisticated mechanism where the same family of proteins can tip the balance towards one or the other endpoint. The mechanism through which wild-type *ras* can reverse the oncogenic properties of the mutant counterpart is of outmost importance and remains to be elucidated.

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