Tyrosine Phosphorylation Pathways and Oncogenesis

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The mitogen-activated protein kinase (MAPK) cascade is a major signaling system by which cells transduce extracellular signals into intracellular responses. A member of this family, extracellular signal-activated kinase (ERK), was shown to play a crucial role in proliferation and apoptosis. Since the development of tumors often results from the alteration of different signal transduction pathways, ERK may play an important role as a biomarker in tumor diagnosis and prognosis. ERK has been implicated in cell initiation, tumor promotion and progression, invasion, metastasis, and the regulation of apoptosis and angiogenesis, events that are essential to the development of a metastatic tumor. The possibility of using ERK as an early molecular epidemiologic biomarker of human carcinogenesis cannot be overlooked.

Our previous experiments have shown that a flavonoid-rich solution, Flavin7, was able to diminish kidney tumor growth in vivo. The effects of Flavin7 on the MAPK signaling pathway were determined in immortalized mouse proximal tubule cells by determining cell viability, flow cytometric analysis, luciferase assays and Western blots. At a nontoxic dose, Flavin7 markedly reduced phosphorylation of ERK and inhibited activity of its downstream targets such as Elk1 and CREB via inhibition of the ERK-kinase MEK1. Further in vivo investigations may determine the potential role of Flavin7 in the treatment of malignancies.

1. Tyrosine phosphorylation pathways and oncogenesis

1.1. Introduction

The mitogen-activated protein kinase (MAPK) cascade is a major signaling system by which cells transduce extracellular signals into intracellular responses. Many steps of this cascade are conserved, and homologues have been discovered in different species (reviewed in 1). MAPK family members all have the unique feature of being activated by phosphorylation on threonine and tyrosine residues by an upstream dual-specificity kinase (reviewed in 1), while MAPK phosphorylate substrates on serine or threonine adjacent to proline residues are proline-directed protein kinases. These cascades are found in all eukaryotic organisms and consist of a three-kinase module that includes a MAPK, which is activated by a MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK), which in turn is activated by a MEK kinase (MEKK).

Mitogen-activated protein (MAP) kinases were first characterized as agents phosphorylating MAP2 and the model substrate myelin basic protein in the late 1980s (2-4). The first three mammalian MAP kinases, ERK1, ERK2 and ERK3 were cloned in the early 1990s. It has become clear that ERK1 and ERK2 are among the protein kinases most commonly activated in signal transduction pathways. They are particularly linked to cell proliferation, but have important roles in many other events (2, 3). In mammalian cells, the ERK1 and ERK2 (often referred to as p44 and p42 MAP kinases), are the archetypal members of the MAPK family. Besides those, the family includes 4 isoforms of p38 MAP kinase (α, β, γ, and δ); 3 genes encoding 10 or more splice variants of the c-Jun N-terminal kinase/ stress-activated protein kinases (JNK/ SAPKs); and at least 3 forms of ERK3 apparently coded by 2 genes; ERK4; ERK5 and ERK7 (4).

The JNK/SAPKs group was discovered as a cycloheximide-activated praline-directed kinase that binds to and phosphorylates the N-terminal sites of c-Jun (5, 6). cDNA cloning identified three genes encoding the 46- and 54-kDa isoforms of JNK/SAPK (7, 8). These enzymes are activated by UV, antibiotics, cytokines, other environmental stresses and, though to a lesser degree, by growth factors. JNK/SAPKs are important for cytokine biosynthesis and are involved in cell transformation, stress responses, and apoptosis. JNK1 and JNK2 are required for apoptosis in distinct regions of the brain, and JNK1 is also required for proliferation and differentiation of thymocytes (reviewed in 4).

The p38 subgroup was discovered as a lipopolysaccharide-induced tyrosine phosphoprotein. Four p38-like MAP kinases are known so far. They are often activated by cellular stresses and are also referred to as stress-activated protein kinases. Besides their roles in the cytokine biosynthesis, they have been implicated in many other events such as muscle differentiation and B cell proliferation (reviewed in 4).

Since MAP kinases represent an

important pathway for transducing external stimuli to internal signals in cells, determining their possible role as early biomarkers or prognostic factors may offer a promising method for the treatment and prognosis of different cancerous diseases. Though, in contrast to the *in vitro* data, there are only a few and partly contradictory reports on the expression and activity of ERK1/ERK2 in clinical tumor tissues, recent studies have investigated ERK in different human malignancies. According to these latest results, ERK activation is not unambiguously an advantage or a disadvantage for patients with cancerous diseases. ERK has been shown to trigger cell proliferation and survival in normal cells, as well as in tumor cells. Thus, the impact of ERK activation/proliferation on becoming disease-free and on overall survival depends on the tumor type.

ERK has not yet been tested in animal models as an early molecular epidemiologic biomarker of carcinogenesis, but the potential of ERK as an early biomarker of human tumor development cannot be overlooked.

1.2. ERK in carcinogenesis

1.2.1. ERK Signaling Pathways

1.2.1.1. Activation Pathways

MAP kinases are regulated by phosphorylation cascades. Two upstream protein kinases activated in series lead to activation of a MAP kinase, and additional kinases may also be required upstream of this three-kinase module. MAP kinase kinases are dual-specificity enzymes that can phosphorylate hydroxyl side chains of serine/threonine and thyrosin residues in their MAP kinase substrates. In spite of their ability to phosphorylate proteins on both aliphatic and aromatic side chains, the substrate specificity of the known MAP kinases is very narrow (reviewed in 5).

There are several characteristics of MAP kinases that result from their activation by kinase cascades. First, the intermediates provide distinct mechanisms for detecting inputs from other signaling pathways to enhance or suppress the signal to the MAP kinase. Another is signal amplification, which can occur if each successive protein in the cascade is more abundant than its regulator, thus, the signal may be amplified at both steps within the module.

Another feature of MAP kinase cascades derives in part from the dual phosphorylation of the MAP kinase by the MEK. In the case of ERK1/ERK2, the kinases are phosphorylated on tyrosine before threonine is phosphorylated. The result is the establishment of a threshold (reviewed in 7).

1.2.1.2. Control of ERK signaling

MAPK plays an important role in transmitting the signals from receptors on the cell membrane to cytoplasmic targets and downstream kinases and nuclear targets. Thus, regulation of the subcellular localization of MAPK is important for controlling MAPK signaling.

Regulatory mechanisms of subcellular distribution of the ERK1/ERK2-type MAPK have been extensively investigated. In quiescent cells, ERK1/ERK2 is largely cytoplasmic and translocates to the nucleus upon stimulation. ERK1/ERK2 does not have an authentic signal

sequence for nuclear import or nuclear export. As ERK1/ERK2 is small enough to enter the nuclear pore through passive diffusion, it is thought that there are anchor proteins which tether ERK1/ERK2 in the cytoplasm. MEK1/MEK2, an upstream kinase of ERK1/ERK2, localizes to the cytoplasm because of its nuclear export sequence in its N-terminal region. The binding of ERK1/ERK2 to MEK1/MEK2, which forms an ERK/MEK heterodimer, results in the cytoplasmic retention of ERK1/ERK2, and nuclear translocation of ERK1/ERK2 is accompanied by the dissociation of ((ERK1/ERK2)/(MEK1/MEK2)) complex. Phosphorylation of ERK1/ERK2 by MEK1/MEK2 is necessary and sufficient for the dissociation of (ERK1/ERK2)/(MEK1/MEK2) complex (reviewed in 8).

There are three independent mechanisms for nuclear translocation of ERK1/ERK2: passive diffusion of a monomer, active transport of a dimer, and importin-independent transport. Phosphorylated ERK2 forms a dimer with phosphorylated or unphosphorylated ERK2. Moreover, disruption of dimerization by mutagenesis of ERK2 reduces its ability to accumulate in the nucleus, indicating that dimerization is important for its translocation to the nucleus (9). Although it is unclear whether these three mechanisms are equally important in nuclear translocation of ERK1/ERK2, active transport does not appear to be a major mechanism for nuclear import of ERK1/ERK2, as a recent study with live cell imaging has shown that the movement of ERK1/ERK2 upon stimulation can be explained for the most part by energy-independent mechanisms (10).

The nuclear accumulation of ERK1/ERK2 is temporary and ERK1/ERK2 must relocalize to the cytoplasm to prepare for the next stimulation. Nuclear export of ERK1/ERK2 involves a MEK1/MEK2-dependent, active transport mechanism: MEK1/MEK2 is shuttling between the cytoplasm and the nucleus, and carries ERK1/ERK2 out to the cytoplasm by using the nuclear export activity (8). While ERK1/ERK2 phosphorylates and activates several nuclear targets, including transcription factors, part of the activated ERK1/ERK2 localizes to the cytoplasm and phosphorylates cytoplasmic targets. Thus, the regulation of the spatial direction of ERK1/ERK2 signaling is essential.

According to the sequential-predictive model of carcinogenesis shown on Figure 1, the development of tumors from the first initiated cell to metastasis includes at least 5 steps: initiation, promotion, progression, invasion, and metastasis (11). Activation of MAP kinases during tumorigenesis is very frequent, and could play an important role in each of the above five steps.

1.2.2. ERK in the phases of carcinogenesis

1.2.2.1. Initiation

Mutations of the Ras family of proto-oncogenes are present in 30% of human tumors, contributing to the development of cancer. The most widely studied effectors for Ras signaling are the Raf serine/threonine kinases. Ras

promotes Raf association with the plasma membrane, where other events facilitate Raf activation. Raf then phosphorylates and activates the MEK1 and MEK2 dual specificity kinases. Activated MAP kinases translocate to the nucleus where they regulate gene expression by modulating transcription factors (reviewed in 12).

Mouse multistage carcinogenesis protocols include treatment of the mouse skin at the primary step of initiation with chemical carcinogens, which results in mutations of Ha-ras at specific codons that activate its transforming properties. Other changes in Ha-ras activity result from its amplification. The alterations in Ha-ras correlate with an increase in mitogenic signaling, represented by elevated ERK activities in cells cycling in serum. These signals can be reproduced partially if a human allele of Ha-ras is introduced in squamous cell carcinoma cells. Similarly, they can be inhibited by small synthetic inhibitors of the MAP kinase cascade (reviewed in 13).

1.2.2.2. Promotion

The cell proliferative stages of tumor promotion involve the removal of an initiated cell from growth suppression by neighboring cells, which would effect the reduction of gap junctional intracellular communication and the activation of mitogenic intracellular pathways. Intracellular events modify the intracellular proliferative steps of tumor promotion. The intracellular pathways typically activated by a proliferative stimulus are the extracellular response kinases/ERK (14).

Chemicals, such as polycyclic aromatic hydrocarbons (PAH) that contribute to the promoting stages of cancer could also alter these signaling pathways. Benzo(a)pyrene caused a rapid increase in phosphorylation of ERK in an experiment (15). The aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin was also shown to induce the immediate activation of ERK (16).

The recent study of de Lédinghen et al examined transformed and non-transformed hepatocytes to determine the effects of cell transformation on the cyclooxygenase 2 (COX2) expression and to shed light onto the mechanisms leading to COX2 expression in transformed cells. According to this study, the tumor promoters phorbol 12-myristate 13-acetate (PMA) and chenodeoxycholic acid induced COX2 expression in transformed, but not in non-transformed cells. PMA-induced COX2 expression in transformed cells resulted from an induction in COX2 mRNA, and was dependent on ERK, p38 mitogen-activated protein kinase and phosphatidylinositol-3-kinase. The data demonstrated that cellular transformation failed to lead to the induction of COX2 expression in hepatocytes, however, the conditions rendered hepatocytes susceptible to COX2 induction from tumor promoters by post-transcriptional mechanisms. The study has proven that COX2 is a target gene downstream of the MAPK signaling cascades in hepatocytes (17).

Arsenic is a common environmental and occupational pollutant and a well-known human carcinogen that causes cancers in many human organs. It is generally

acknowledged that arsenic does not act via a classic genotoxic or mutagenic mechanism, because it is not a direct mutagen. On the other hand, evidence has shown that arsenic shares many properties with tumor promoters by inducing intracellular signal transduction, activating transcription factors and changing the expression of genes involved in promoting cell growth, proliferation, and malignant transformation. Arsenic-induced MAPK signal transduction leads to activation of transcription factors such as AP-1 and nuclear factor-kappa B. These processes are associated with the carcinogenity of arsenic (18, 19).

1.2.2.3. Progression

The basement membrane forms a cellular support for tumors, and is made up of a complex mix of extracellular matrix proteins. MAPK enzymes have been shown to be involved in regulating the proteolytic enzymes that degrade the extracellular matrix. Therefore, these enzymes have been implicated in the progression and invasion of cancer besides their involvement in normal tissue remodeling, wound healing, and angiogenesis (reviewed in 20).

Most normal cells depend on environment-specific factors to maintain their viability and to prevent them from surviving in nonphysiologic sites (20). Defective apoptosis facilitates tumor progression, because the cells can ignore restraining signals from their neighbors, survive detachment from the extracellular matrix, and persist in a hostile environment. In the last few years, a large body of evidence has indicated the involvement of the MAPK family in the regulation of apoptosis. The activation of JNK/SAPK is generally associated with the promotion of apoptosis, while activation of ERK was shown to inhibit apoptosis (reviewed in 20). Studies have shown that ERK acting downstream of B-Raf inhibits caspase activation following cytochrome c release from the mitochondria. It is also possible that ERK phosphorylates a proapoptotic Bcl-2 family protein, Bad, which is known to influence the mitochondrial membrane integrity and release of cytochrome c, by associating with Bcl-2 and Bcl-xL and thus inhibiting their anti-apoptotic function. Phosphorylation of Bad induces its association with different proteins, thus sequestering it in the cytosol and away from mitochondria (reviewed in 20). These data suggest that ERK plays a major role in protecting the cell from undergoing apoptosis, and that this impairment of apoptosis could help cells survive during progression, invasion, and metastasis.

1.2.2.4. Invasion

Disruption of the integrity of the basement membrane is a key histological marker of a tumor's transition to invasive carcinoma. In metastatic cells, ERK activity was shown to be higher when compared to non-metastatic cancer cells (21, 22). Persistent activation of ERK in malignant cells can lead to enhanced induction of proteolytic enzymes and this could lead to extracellular matrix and basement membrane degradation, allowing the cancer cells to invade into surrounding tissues and metastasize. Recent studies

have shown that significantly increased active ERK was found in primary breast tumors compared to their adjacent matched normal breast tissues and this increased ERK activity correlates with enhanced nodal metastasis (21, 22).

There is abundant evidence to show that receptor tyrosine kinases are involved in the development and progression of tumors. In addition to promoting mitogenic responses in target cells, these receptors are also capable of regulating cellular functions that are involved in the acquisition of an invasive phenotype such as the modulation of cellular attachments and the proteolysis of extracellular matrix and migration (reviewed in 20). Many growth factors have been reported to stimulate cell migration, through activation of receptor tyrosine kinase involving Ras/ERK signal pathways (23, 24).

Previous studies indicated that ERK activates the cell's motility machinery by enhancing myosin light-chain kinase activity leading to increased myosin light-chain kinase phosphorylation and enhanced cell migration. Different groups have shown that persistent activation or prolonged nuclear retention of activated ERK induces cell motility (25, 26). These findings may explain how growth factors, cytokines, or integrins that activate MAPK can influence cell motility on extracellular matrix during tissue remodeling as well as tumor cell invasion.

1.2.2.5. Metastasis

Metastasis consists of a series of sequential steps including the shedding of cells from a primary tumor in the circulation, the survival of the cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth and vascularization of the metastatic tumor. This process involves the coordination of several signal-transduction pathways that allow cancer cells to proliferate, remodel their surrounding environment, invade to distant sites, and reestablish the tumor. Recent data have shown that ERK plays a major role in inducing proteolytic enzymes that degrade the basement membrane, enhance cell migration, initiate several pro-survival genes, and maintain growth (reviewed in 20). The findings described at the mechanisms of invasion also suggest that ERK can also affect the process of metastasis by direct activation of the intracellular motility machinery.

Due to the central role that the ERK has in the connections between different signal transduction pathways, ERK seems to play a role in every step needed in the development of a metastatic cancer. Further *in vivo* experiments are needed to refine the potential of ERK as an early molecular epidemiologic biomarker.

1.2.3. Prognostic role of ERK in different tumors

1.2.3.1. ERK in breast tumors

With respect to breast tumors, earlier studies suggested an involvement of activated ERK in carcinogenesis and progression. Sivaraman et al reported an increased enzymatic MAPK activity in cytosols from 12 mammary carcinomas as compared to normal tissues or benign lesions (21). Salh et al found increased ERK1 and ERK2 amounts after

immunoprecipitation relative to normal tissue samples, though ERK2 staining intensity in immunohistochemistry was actually reduced in tumor cells (27). A recent study (28) on 148 mammary carcinoma tissues of 120 cases with follow-up data used Western blot analysis and immunohistochemistry to determine the expression of ERK1/ERK2 and their phosphorylated forms in the investigated histological samples. In this study, high phosphorylated ERK1 expression determined by immunoblots correlated significantly with a low frequency of recurrences and infrequent fatal outcome, and was an independent indicator of a long relapse-free period and overall survival in multivariate analysis. By immunohistochemistry, strong phosphorylated ERK staining in tumor cells was associated with early stages, negative nodal status, and long recurrence-free survival. According to these results, activation of ERK1 and ERK2 is associated with a better prognosis for the patient. Furthermore, in the case of breast cancer, survival and proliferation seem to be independent of each other.

1.2.3.2. ERK in colorectal carcinomas

It is known that the ERK pathway is one of the most important for cell proliferation (reviewed in 29), and several key growth factors and proto-oncogenes transduce the signals that promote growth and differentiation through this cascade. Activation of this signaling pathway is important in intestinal epithelial differentiation. There is growing evidence that activation of the ERK pathway is involved in the pathogenesis, progression, and oncogenic behavior of human colorectal cancer (30).

A study, using the 1,2-dimethylhydrazine-induced colon carcinoma model in rats demonstrated that the activities of ERK are highly increased during colonic carcinogenesis *in vivo* (31). However, a more recent study conducted by the same research group on human colorectal tissue samples and their adjacent normal mucosa samples found elevated ERK activities only in a subset of human colorectal cancers (32). Namely, ERK activity in colonic carcinomas was 3.8±1.3-fold greater than in matching normal mucosa. This induction was still less than the 29-fold induction of ERK activity seen in 1,2- dimethylhydrazine-induced carcinomas in rats (32). The difference seen in animal model and human colorectal cancer samples may be explained by genetic and dietary factors which play an important role in human colorectal carcinonogenesis.

On the other hand, a more recent study conducted by Nemoto et al found increased expression of phosphorylated ERK1/ERK2 in colon cancer samples (33). These findings are in accordance with the result of a previous report (34), but not with others (35, 36). The reason for the inconsistency is not clear, but indicates that besides the MAPK signaling pathway, other mechanisms may also be involved in the development of human colorectal carcinomas. Thus, in colorectal cancers, ERK1/ERK2 cannot yet be used as an early biomarker.

1.2.3.3. ERK in prostate cancer

In early MAPK studies, elevated levels of MAPK have been detected in prostate carcinomas using immune-complex kinase assays on tumor tissue homogenates (37). However, analysis of tumor homogenates may be problematic, because non-neoplastic cells are included in the assessment of protein activity. Thus, later studies have focused on phosphorylation-specific antibodies as a means to test for activation of specific signal transduction proteins at the cellular level.

Gioeli et al have examined 82 primary and metastatic prostate tumor specimens for the presence of phosphorylated ERK. High levels of activated ERK were observed in high-grade and advanced-stage tumors. These observations, combined with previous reports demonstrating increased expression of cell proliferation markers, suggest that activation of ERK is linked to cell proliferation in prostate cancer (38). Data also suggest that elevated ERK activation may also be important in the growth of androgen-independent prostate cancer. Analysis of several patients' tumor samples showed high levels of activated ERK only after androgen ablation treatment, suggesting that ERK is activated in hormone refractory tumors (38).

Another study investigated the expression of ERK1 and mitogen-activated protein kinase phosphatases (MKP-1) by immunohistochemistry in 50 cases of high-grade prostatic intraepithelial neoplasia, thought to represent the precursor of prostate cancer (39). Protein overexpression of the MAP kinases was found in all cases compared to a normal prostate. These findings suggest that MAP kinases, even in prostatic intraepithelial neoplasia, may shift the balance between cell proliferation and death. When they are expressed, the pathways that lead to apoptosis may be inhibited (39).

1.2.3.4. ERK in verrucous and squamous cell carcinomas of the upper digestive tract

Lessard et al studied 17 verrucous carcinomas and 10 squamous cell carcinomas of the upper aerodigestive tract for ERK1/ERK2 expression by means of immunohistochemistry (40). In 16 of the 17 verrucous carcinomas, the most basally situated layers of epithelial cells showed minimal ERK1 and ERK2 staining. This was in contrast with the moderately differentiated squamous cell carcinomas, in which the tumor cells, even the ones most basally situated, stained with ERK1 and ERK2. It is unclear why there was such exquisite localization of the staining. Possibly, there is a block in synthesis of ERK1 and ERK2 in the proliferating cells of verrucous carcinoma (40). It is also possible that the differences of staining patterns could be used to help separate verrucous carcinoma and well-differentiated squamous cell carcinoma on small biopsies.

In verrucous and squamous cell carcinomas of the upper digestive tract, the histologic grade was related to the percentage of ERK-positive cells, implying that ERK is a marker of proliferation in these tumors (41).

1.2.3.5. ERK in cervical intraepithelial neoplasia

In the study of Branca et al (42), 302 archival samples (150 squamous cell carcinomas and 152 cervical intraepithelial neoplasia lesions) were subjected to immunohistochemical staining with ERK1 antibodies. There was no significant difference in ERK1 expression in the 3 histological grades of tumor differentiation. However, ERK1 overexpression proved to be a 100% specific marker of cervical intraepithelial neoplasia, and was never found in biopsy specimens without cervical intraepithelial neoplasia. Thus, ERK expression seems to be an early marker of cervical carcinogenesis.

1.2.3.6. ERK in serous ovarian carcinoma in effusions

Givant-Horowitz et al have analyzed 64 fresh-frozen effusions from patients diagnosed with serous ovarian carcinoma using immunoblotting to determine the possible associations between proliferation markers and apoptosis markers, patient age, disease stage, tumor grade, histological grade, chemotherapy status and survival (43). Phosphorylated ERK activity was seen in post-chemotherapy specimens and correlated with better overall survival. The improved prognosis associated with the expression and phosphorylation of ERK may expand the understanding of the biology of ovarian carcinoma, possibly affecting treatment strategies for this malignancy.

1.2.3.7. ERK in human renal cell carcinomas

Oka et al examined whether constitutive activation of the MAPK cascade was associated with the carcinogenesis of human renal cell carcinomas in a series of 25 tumors and in corresponding normal kidneys (44). Constitutive activation of MAPK in tumor tissue, as determined by the appearance of phosphorylated forms, was found in 48% of the cases. The phosphorylation, monitored by SDS-polyacrilamide gel electrophoresis, which is associated with the activation of MAPK, occurred in 50% of the renal tumors. Altogether, 14% of grade 1 tumors, 69% of grade 2 tumors, and 40% of grade 3 tumors showed MAPK activation (44). These results suggest that constitutive activation of MAPK may be associated with the carcinogenesis of human renal cell carcinomas.

1.2.3.8. ERK in hepatocellular carcinomas

Twenty-six hepatocellular carcinomas and their adjacent normal liver tissues were analyzed by Ito et al for the activation/phosphorylation of ERK and its associated gene expression by immunoblotting and immunohistochemistry (45). ERK was activated in 58% of the cases and its expression level was significantly higher in hepatocellular carcinomas than in adjacent non-cancerous lesions.

The involvement of ERK activation in the clinico-histopathological features of hepatocellular carcinomas was assessed in the study as well. Activated ERK was elevated along with tumor size and was

significantly higher in hepatocellular carcinomas over 20 mm in diameter than in those under 20 mm. As it is well known, hepatocellular carcinomas exhibit changes in their characteristics when they reach approximately 20 mm in size (reviewed in 45). Therefore, the observed activation of ERK may be related to those changes of characteristics in the development of hepatocellular carcinomas.

In conclusion, the study suggests that expression of activated ERK in human hepatocellular carcinomas may play a role in multi-step hepatocarcinogenesis, especially in the acceleration of hepatocellular carcinoma *in vivo*.

1.2.3.9. ERK in glial neoplasms

Abnormal growth factor signaling is implicated in the pathogenesis of gliomas. The ERK pathway is a likely target, linking receptor tyrosine kinase activation to downstream serine/threonine phosphorylation events regulating proliferation and differentiation. In the study of Mandell et al (46), immunohistochemical detection of phosphorylated/activated ERK on different glial neoplasms permitted visualization of spatially discrete cellular patterns of ERK activation, compared to the relatively uniform expression of total ERK protein. The astrocytic tumors, regardless of grade, had the highest overall degree of ERK activation, whereas oligodendrogliomas had the least. Anaplastic progression of oligodendrogliomas resulted in a larger number of cells with active ERK. Within glioblastomas, microvascular hyperplasia and necrosis were associated with ERK activation in adjacent tumor cells. In addition to spatial patterns of intratumor paracrine signaling, a possible cell cycle-associated regulation was detected: mitotic and actively cycling tumor cells showed diminished activation relative to cells in G0 phase. Although ERK activation was not restricted to neoplastic glia, consistent patterns of selective activation in tumor cells suggest that sustained activation may contribute to the neoplastic glial phenotype (46).

1.2.4. ERK as prognostic factor/biomarker of cancer

When the findings described above are considered together, ERK1 as an indicator of an activated ERK signaling pathway seems to be an early marker in a wide range of epithelial tumors, including cervical intraepithelial neoplasia, breast cancer, prostate cancer, verrucous and squamous cell carcinoma of the upper aerodigestive tract, renal cellular carcinoma, hepatocellular carcinoma, and glial neoplasia. However, there might be differences between the different histologic types of human cancer, as suggested by the findings of Loda et al, who found ERK1 and ERK2 overexpression in the early phase of prostate, colon and bladder carcinogenesis, with progressive loss of expression with higher histologic grade and in metastases (47).

1.3. Flavonoids and ERK activation in carcinogenesis

1.3.1. Flavonoids in carcinogenesis

Flavonoids, found in great quantity in fruit extracts, are secondary metabolites of superior plants exhibiting antitumor effects. They are known to exert antioxidant and antiproliferative effects on tumor cells (48). Recent studies have speculated that the classical antioxidant activity of flavonoids is unlikely to be the sole explanation for their cellular effects. This hypothesis is based on several lines of reasoning: i) flavonoids are extensively metabolized *in vivo*, thus, their redox potentials are significantly altered (49), and ii) the concentrations of flavonoids and their metabolites accumulated *in vivo* are lower than those of antioxidant nutrients (8). Investigations have indicated that flavonoids may selectively interact with the MAPK signaling pathway due to their ability to inhibit tyrosine kinase activity (49, 50).

A natural compound, Flavin7 (F7), composed of the extracts from seven different fruits, was investigated in our kidney tumor animal model (51). Ne/De tumor cells were transplanted underneath the renal capsule of 6-to 8-week-old Fisher344 rats and animals were treated with human dose-equivalent F7 solution according to the manufacturer's instructions. After two weeks of treatment the rats were sacrificed and tumor growth was determined. F7 significantly ($P<0.05$) reduced tumor growth *in vivo*.

Accordingly, the aim of this study was to determine whether F7 influences the ERK signaling pathway in immortalized mouse renal proximal tubule cells.

1.3.2. Materials and Methods

1.3.2.1. Cell culture

TKPTS cells where TKPTS cell line was obtained from Dr Bello-Reuss and grown in 5% CO_2 atmosphere at 37ºC as described by di Mari *et al.* (52). Parallel sets of logarithmically growing cells were treated with 50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7 (total energy content 9.7 kJ, protein 0.07 g, fat 0.02 g, carbohydrates 0.16 g, total polyphenol 85 mg (flavonoid 75 mg, resveratrol 0.16 mg) 8 v/v% alcohol per 10 ml solution) (Crystal Institute Ltd, Eger, Hungary) per 2 ml cell culture medium.

1.3.2.2. Cell survival and FACS analysis

TKPTS cells were treated with various doses of Flavin 7 (50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7/2 ml culturing medium). Viable cell count was determined by trypan blue (Sigma Chemical Co, St. Louis, MO, USA) exclusion in a hemocytometer after 24 hours of treatment. In addition, TKPTS cells treated with 100 μl F7 were collected for FACS analysis 24 hours after treatment. Briefly: TKPTS cells were collected after trypsinization and fixed in 70% ethanol overnight. Afterwards, RNAse treatment cells were incubated with 5μg/ml PI and analyzed with a Becton Dickinson FACSCalibur analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). The cell cycle profile was analyzed using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

1.3.2.3. Luciferase assay

pCRELuc plasmid containing four direct repeats of consensus CRE binding sites

(Stratagene, La Jolla, CA, USA) was transiently transfected into TKPTS cells by using the GenePorter2 reagent (GenLantis, San Diego, CA, USA) together with a β-galactosidase plasmid (Promega, Madison, WI, USA) as described elsewhere (53). Similarly, the pFR-Luc reporter plasmid together with the pFA2-Elk1 trans-activator plasmid (Stratagene, La Jolla, CA, USA) were transiently transfected into TKPTS cells. Twenty-four hours after transfection cells were treated with Flavin 7 for various time points (50μl, 100μl, 200 μl and 300 μl F7/2 ml culturing medium for 12 h). Luciferase activity was determined by using a Luciferase Assay Kit (Promega, Madison, WI, USA) while β-galactosidase

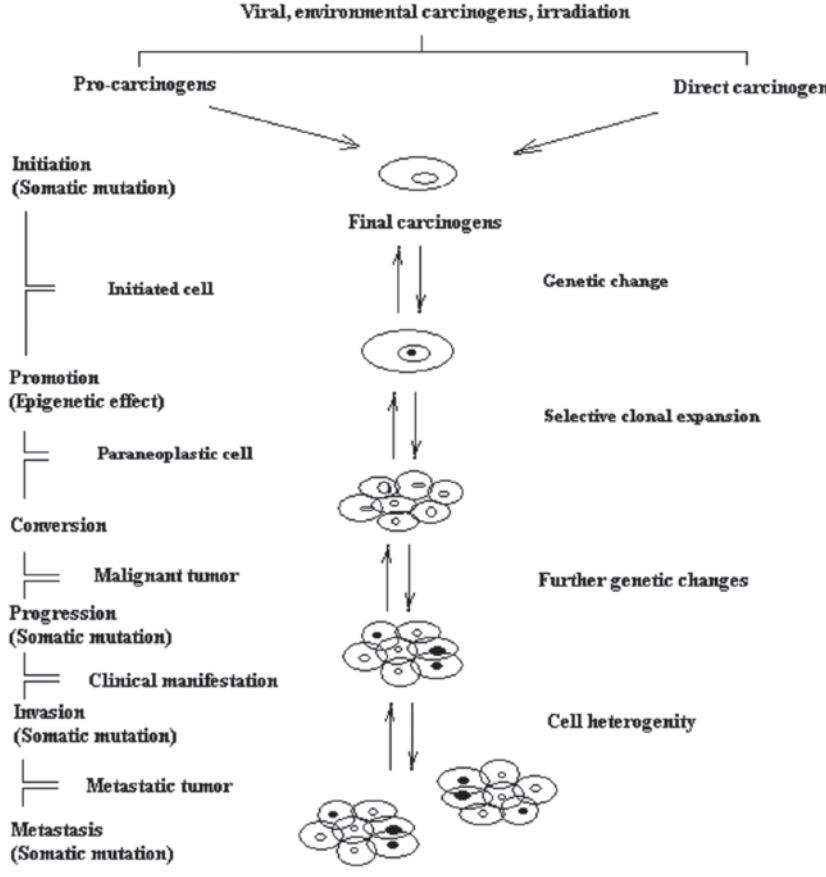

Figure 1. Sequential-predictive model of carcinogenesis (reproduced with permission from the authors, 12).

was determined under control conditions and 18 hours after F7 treatment. The relative luciferase activity was measured and was normalized to the amount of activity detected for the co-transfected β-galactosidase plasmid (Invitrogen, La Jolla, CA, USA).

1.3.2.4. Western blots

Sets of logarithmically growing cells were treated with 100 μl F7 per 2 ml cell culture medium. Thirty minutes, 1 h, 2 h and 6 h after F7 treatment the medium was discarded, the cells were rinsed with 1xPBS and collected in a radioimmunoprecipitation assay (RIPA) buffer containing 50 μl/ml proteinase inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA), 100 mmol/ml sodium orthovanadate (Sigma Chemical Co.) and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) as described elsewhere (7). Protein content was measured using the Bio-Rad Protein Determination assay (Bio-Rad, Hercules, CA, USA) and 50 μg protein was blotted onto polyvinylidine fluoride membranes. The membranes were then hybridized with phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 (Cell Signaling Technology, Beverly, MA, USA), phospho-MEK (Ser217/221) and MEK (Cell Signaling Technology) primary antibodies overnight in 5 ml 5% milk solution according to the manufacturer's instructions. After washing, the membranes were hybridized with a horseradish-peroxidase (HRP)-conjugated secondary antibody in 2 ml 5% milk solution for 45 min. Bands were visualized by using the enhanced

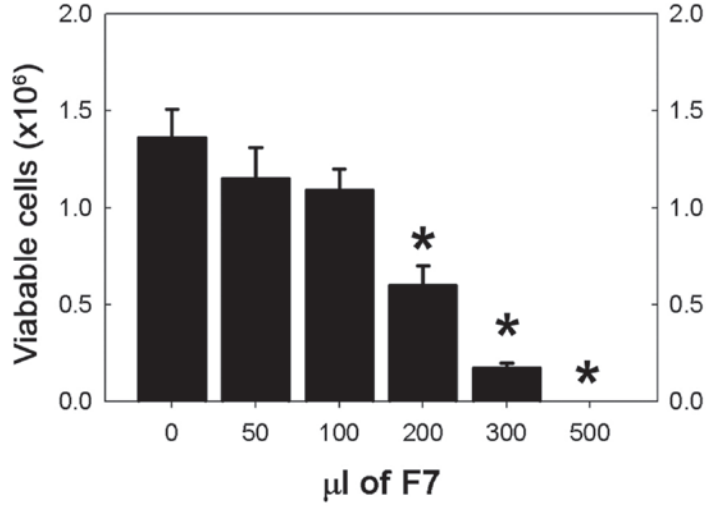

Figure 2. Effect of F7 treatment on survival of TKPTS cells. Cell count was determined by trypan blue exclusion (mean±S.D., n=3, *P<0.001 compared to untreated control)

chemiluminescence (ECL) method (Amersham, Piscataway, NJ, USA) and quantified by densitometry (UnScan-It 6.1; Silk Scientific, Orem, UT, USA).

1.3.2.5. Statistical analysis

Statistical differences between the treated and control samples were determined by Student's paired *t* test. Differences between means were considered significant when $p<0.05$. Analyses were performed by using the SigmaStat 3.5 software package.

1.3.3. Results

1.3.3.1. Effects of F7 treatment on cell viability

Treatment of the TKPTS cells with 50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7/well showed that 200 μl, 300 μl and 500 μl F7 significantly reduced the number of cells in the culture while the 50 μl and 100 μl treatment had no such effects (Figure 2). FACS analysis of the TKPTS cells treated with 100 μl F7 showed that F7 did not change the cell cycle distribution of the cells significantly (Figure 3). Since no cytotoxic effect or change in viability could be seen when applying 50 μl or 100 μl F7, to assure the greatest therapeutic dose possible, we selected the 100 μl F7 dose for our further studies.

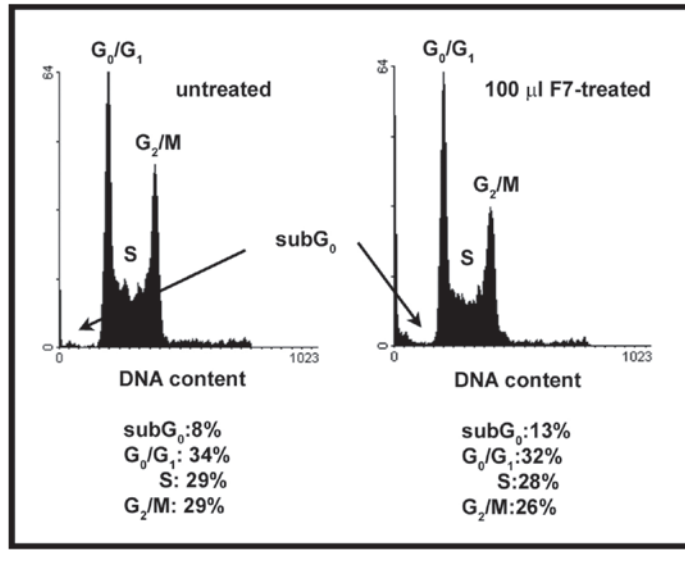

Figure 3. Effect of F7 on the cell cycle progression of TKPTS cells. TKPTS cells were treated with 100 μl F7 for 24 hours and FACS analysis was carried out as described in Materials and Methods. The percentage of cells residing in the different stages of the cell cycle are given. Data are representative of three independent experiments.

1.3.3.2. Effects of F7 treatment on ERK and MEK phosphorylation

Next, we determined whether F7 treatment affected phosphorylation of ERK. As shown in Figure 4, amounts of phospho-ERK were significantly and transiently reduced after F7 treatment in TKPTS cells (Figure 3 A and B). Since ERK is activated through its upstream kinase MEK (54), we were interested whether F7 affects ERK phosphorylation through its kinase. As the results of Western blotting show, F7 attenuates MEK phosphorylation (Figure 5 A and B) similar to ERK. On the other hand, total amounts of ERK and MEK were unchanged.

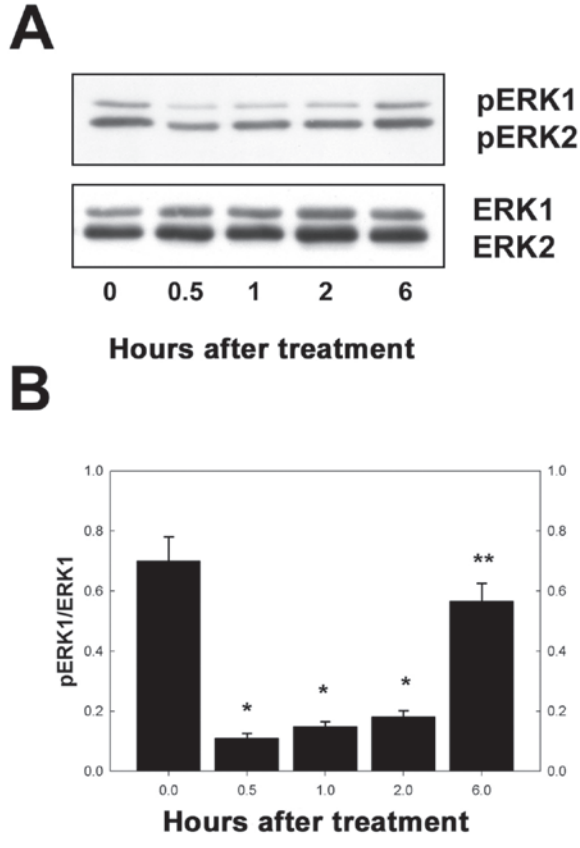

Figure 4. Effect of F7 on ERK phosphorylation in TKPTS cells. (A) TKPTS cells were treated with 100 µl F7 for the times indicated. Phosphorylation of ERK was determined by Western blotting using an antibody that recognizes only the phosphorylated form. As loading controls, total ERK levels were also determined. Results shown are representative of three independent experiments. (B) Densitometry of the Western blots shown in (A). Ratios of pERK1/ERK1 are given (mean±S.D., n=3; *P<0.001 compared to the untreated control, **P<0.001 compared to the 0.5, 1.0 and 2.0 h treatments)

1.3.3.3. Effects of F7 treatment on downstream function of ERK

Activated ERK activates downstream targets that induce target-specific transcription, which is part of the survival signaling (53, 55). Elk1 is one of the downstream targets of ERK, activity of which could be followed by a trans-activating system, where the Elk activator plasmid initiates activity of a luciferase reporter plasmid. Accordingly, TKPTS cells were cotransfected with those plasmids and treated with 50 µl, 100 µl, 200 µl, 300 µl and 500 µl F7/2 ml culturing medium for 18 hours. As seen in Figure 5, Elk1 activity was significantly reduced after F7 treatment, similar to ERK phosphorylation (Figure 4). These observations suggest that ERK function is inhibited by F7 treatment.

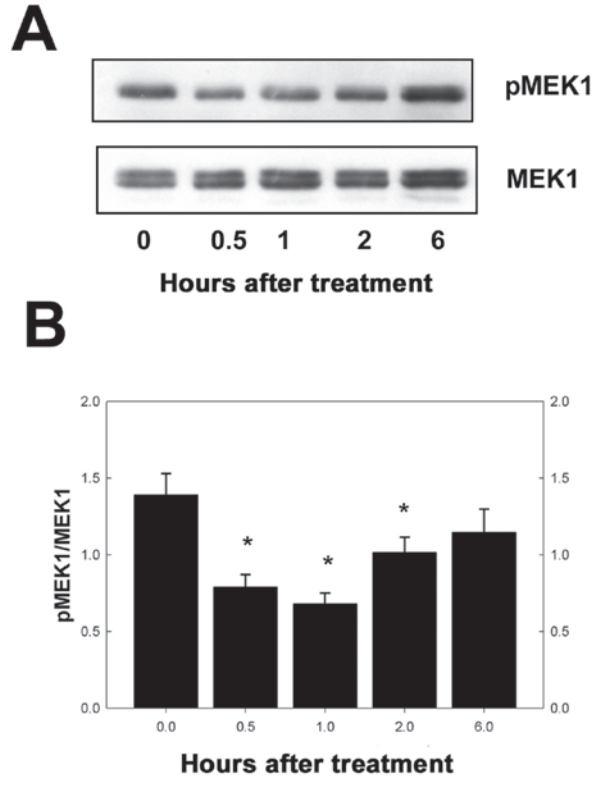

Figure 5. Effect of F7 on MEK1 phosphorylation in TKPTS cells. (A) TKPTS cells were treated with 100 µl F7 for the times indicated. Phosphorylation of MEK1 was determined by Western blotting using an antibody that recognizes only the phosphorylated form. As loading controls, total MEK1 levels were also determined. Results shown are representative of three independent experiments. (B) Densitometry of the Western blots shown in (A). Ratios of pMEK1/MEK1 are given (mean±S.D., n=3; *P<0.001 compared to the untreated control)

The cAMP responsive element binding protein (CREB) is activated through phosphorylation by ERK/p90rsk (53, 56, 57). Activated CREB initiates a series of transcriptional events by binding to the promoters of CREB-responsive genes as part of the survival mechanism (58). To determine whether F7 also affects CREB-mediated transcription, TKPTS cells were transiently transfected with the CREB responsive pCRE-Luc plasmid. As is seen in Figure 6, F7 treatment significantly inhibited CREB-mediated transcription.

1.3.4. Discussion

Flavonoids comprise a large class of naturally occurring polyphenol plant compounds. The human diet usually contains approximately 1 g or more per day of flavonoids, a quantity providing pharmacologically significant concentrations in body fluids (59). Flavonoids were shown not only to inhibit tumor cell growth (51, 59) but also to induce cell differentiation (59). The inhibitory effects of flavonoids on the growth of malignant cells may partly be due to their suppressive effect on kinase

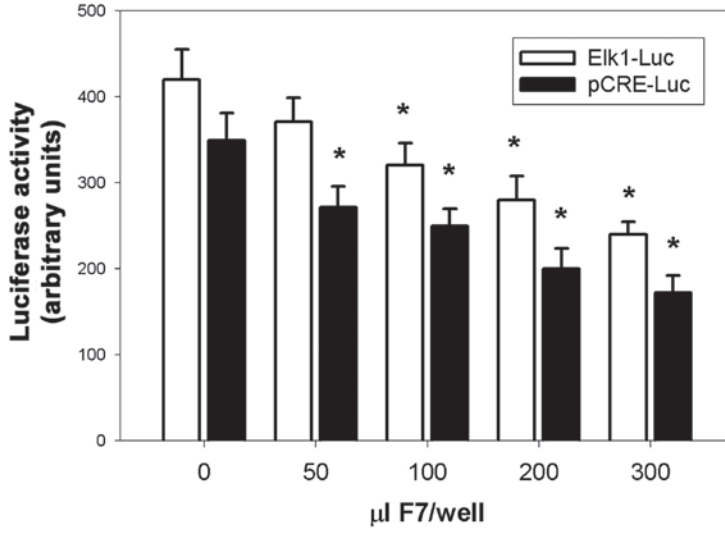

Figure 6. Effect of F7 on downstream function of ERK. TKPTS cells were transiently transfected with either the pFR-Luc reporter plus pFA2-Elk1 trans-activator plasmids together with a β-galactosidase plasmid (open bars) or a pCRE-Luc plus β-galactosidase plasmid (filled bars) as described in Materials and Methods. After 24 hours, cells were treated with F7 for 12 hours and luciferase activity together with β-galactosidase activity was determined. Luciferase activity was calculated as the ratio of luciferase activity normalized to β-galactosidase activity (mean±S.D., n=3; *P<0.001 compared to the untreated control)

activities involved in the regulation of cell proliferation (60, 61).

According to recent data, activation of the MAPK pathway such as ERK signaling is a frequent event in the development of cancerous diseases, though ERK phosphorylation is not unambiguously an advantage or a disadvantage for patients with cancerous diseases (62). The study of Ye *et al.* (63) has shown that the inhibition of the MEK/ERK pathway resulted in a significant enhancement of growth inhibition in MCF-7 breast cancer cells. So *et al.* (64) have demonstrated that citrus flavonoids effectively inhibited proliferation of the human breast cancer cell line MDA-MB-435 *in vitro*, especially when paired with quercetin, which is widely distributed in other foods.

Earlier we reported that F7 treatment was able to reduce the growth of kidney tumor Ne/De transplanted into F344 rats. Furthermore, cancer-related weight loss was also reduced in tumor-bearing animals when treated with F7 (51). The mechanism of this tumor-reducing effect was not investigated, but besides other mechanisms, effects on the MEK/ERK pathway may be suspected.

Our data suggest that F7, being a flavonoid-rich solution, possesses a protein kinase inhibitor activity that might be responsible for the observed inhibition of MEK and ERK phosphorylation in TKPTS cells (Figure 3 and 4). Whether F7 directly acts on MEK or on its upstream kinase(s) needs further investigation. Our data also showed that inhibition of ERK phosphorylation resulted in inhibition of its downstream function, such as Elk1 or CREB activation (Figure 5).

ERK is involved in various cellular processes such as differentiation, proliferation, and survival (62). Interestingly, inhibition of ERK and its function does not affect cell cycle progression as evidenced by FACS analysis (Figure 2). On the other hand, F7 also significantly inhibited CREB activity (Figure 5) probably through inhibition of ERK (53). Since CREB is an important element of survival signaling (7, 53, 57) these results suggest that F7 affects the survival pathway rather than the cell proliferation in renal tubular cells. The mechanism of these effects needs further study.

In conclusion, because of its ability to temporarily inhibit the activation of the MEK/ERK pathway *in vitro* and tumor growth *in vivo*, in case of malignancies which require MEK/ERK activation for survival (65), the natural compound F7 may have merit in the supportive therapy of cancer patients. Therefore, further *in vivo* and *in vitro* investigations are needed to determine the potential role of F7 in the treatment of malignant diseases.

2. Personal experiences

It is a great opportunity in my profession to spend an academic year in the US and get professional experience in laboratory research. My supervisor, Robert L. Safirstein, MD has taken good care of me and made sure that I found my place in the laboratory ever since the first day. My Adviser, Istvan Arany PhD, CSc has done his best to teach me new laboratory techniques like Luciferase assay and

Western blotting and to include me in all the professional and leisure activities of the laboratory personnel. I have done my best to work as hard as possible and achieve some results within this relatively short period for research. I am deeply grateful to Dr. Arany, Dr. Safirstein, and all the laboratory personnel who warmly welcomed me in their everyday work and did not hesitate to help whenever I needed professional support.

I am also truly grateful to my roommate, Rebekah Craig, who did not hesitate to share her apartment with me and welcomed me into her circle of friends. She also taught me a lot about the hospitality of the South and made sure that I felt myself at home no matter where we went together and whom we met. Thanks to her, I met students of different nationalities and forged friendships that will hopefully last forever.

I also need to mention the International Friendship Organization (IFO), a non-profit organization whose primary goal is to welcome foreign students from all over the world in Little Rock, AR and to make them feel at home. They also provide free English lessons to ensure better professional and everyday communication for the foreign students, and, last but not least, they organize different activities for foreign students and Americans, giving them the opportunity to get to know each other and each other's culture.

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