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K-FGF mediated transformation and induction of metastatic potential involves altered ornithine decarboxylase and S-adenosylmethionine decarboxylase expression – role in cellular invasion

Marcus S. Hardin, Rene Mader and Robert A.R. Hurta

Department of Laboratory Medicine and Pathobiology, St. Michael's Hospital, University of Toronto, Toronto, ON, Canada

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Abstract

Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) expression was investigated in NIH-3T3 fibroblasts that secrete K-FGF. Correlations between altered ODC and SAMDC expression and malignant potential were determined. Increased ODC and SAMDC expression was associated with increased expression of both ODC and SAMDC mRNA and enzyme activity levels. Transcriptional and post-transcriptional regulatory mechanisms were found to account for the increased expression of both ODC and SAMDC. Amplification of the ODC gene also played a role. Correlations between the expression of ODC and the invasion ability of the K-FGF overexpressing cells were also found. Additionally, putrescine, which is a cellular polyamine, was found to play a role in determining the nature of the invasive capacity of the K-FGF overexpressing cells. The results of this study which established correlations between alterations in the expression of ODC and SAMDC, the key rate limiting and regulatory activities in the synthesis of cellular polyamines, and malignant potential as a consequence of K-FGF overexpression supports a model which suggests that growth factor modulation of ODC and SAMDC expression is part of the altered growth regulatory program associated with cellular transformation and malignant progression. (Mol Cell Biochem **233**: 49–56, 2002)

Key words: K-FGF, ornithine decarboxylase, S-adenosylmethionine decarboxylase, altered expression

Introduction

Metastasis is a very complicated process whereby tumour growth at sites distant from the primary neoplasm can result. Many of the modified properties of metastatic populations of cells may be controlled by alterations in the expression of a number of key genes [1]. These genes are usually critical determinants of a number of diverse functions. These functions may include cellular proliferation, cellular differentiation, cell-to-cell communication and cellular motility and invasion. Growth factors can also play important and critical roles in cellular transformation events through mechanisms of autocrine stimulation [2]. Tumour cells have the ability to produce and to release a number of potent mitogenic growth factors which can contribute to the establishment of the malignant state [2, 3].

One of these growth factors, k-FGF has been shown to possess potent transforming activity [3, 4] and the k-fgf gene has been found to be amplified in a variety of human tumours [5, 6]. Transformation and amplification of the K-fgf proto-oncogene in NIH-3T3 cells has also been shown to result in induction of metastatic potential [7]. Changes in cell motility, invasion and malignancy induction can all occur following over-expression of the K-fgf gene [8]. K-fgf has also been implicated in the transformation mechanisms leading to human cancer development [5, 9]. K-FGF can affect cellular biological properties through both autocrine and intracrine mechanisms [8].

Address for offprints: R. Hurta, Department of Biology, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, Canada, C1A 4P3 (E-mail: rhurta@upei.ca)

Cellular polyamines, namely putrescine , spermidine and spermine, are important for mammalian cellular proliferation [10]. The two key regulatory activities in the biosynthesis of polyamines are ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC). Ornithine decarboxylase catalyzes the conversion of ornithine to putrescine. Mammalian ODC is a highly regulated activity and ODC expression is controlled by a variety of agents capable of stimulating cellular proliferation [11–13]. ODC expression is subject to complex regulation that can occur at transcriptional, translational, post-transcriptional and post-translational levels [14–18]. Mammalian SAMDC is also a highly regulated enzyme whose levels can fluctuate depending upon the growth status and the intracellular polyamine levels of cells [10]. SAMDC catalyzes the formation of decarboxylated S-adenosylmethionine which acts as the aminopropyl donor for the biosynthesis of the polyamines, spermidine and spermine [19]. The expression of SAMDC and ODC has been well documented to increase in cells which have been stimulated to proliferate [10, 20].

Alterations in certain cellular polyamine levels have been associated with alterations in the expression and regulation of a number of genes including cell cycle associated genes, cytoskeletal genes, and matrix metalloproteinases , amongst others [21–23]. Alterations in polyamines have also been suggested to be key determinants in inducing malignant transformation and in mediating oncogene expression [24, 25]. Over-expression of either ODC or SAMDC has been suggested to confer a more invasive phenotype on cells [26–28]. Furthermore, intracellular putrescine has been suggested to modulate the *in vitro* invasive capacity of cells [29].

This investigation addressed questions concerning ODC and SAMDC expression and regulation in response to growth factors. We tested, for the first time, whether K-FGF expression could regulate the expression of ODC and SAMDC. This study demonstrates that K-FGF over-expression and amplification can alter the expression of both ODC and SAMDC in cells. This study further demonstrates that these alterations contribute to the altered cellular phenotype of these K-FGF over-expressing cells including their cellular invasive capacity.

Materials and methods

Cell lines and culture conditions

Cell lines were grown in alpha minimal essential medium (Life Technologies) containing 10% fetal bovine serum (Hyclone) and supplemented with antibiotics. The dC2, NIH-3G (3G) and the NIH-3G-20 (3G-20) cell lines have been previously described [8]. Briefly, the 3G and 3G-20 cell lines were derived as independent clones from NIH-3T3 cells that were transformed by transfection with the K-fgf gene [7, 8]. They secrete higher levels of K-FGF in the medium than the parental cell line [8]. The dC2 cell line is a NIH-3T3 derived cell line transfected with the vector used to introduce K-fgf into NIH-3T3 cells but lacking the K-fgf coding sequence [7]. The dC2 cell line is not tumourigenic, whereas the 3G and the 3G-20 cell lines are moderately and highly metastatic cell lines, respectively [8].

Nucleic acid analysis

Genomic DNA was prepared by a rapid genomic DNA isolation method using the DNAzol reagent (Life Technologies) according to the manufacturer's instructions and then Southern blot analysis was carried out as described previously [30]. Total cellular RNA was isolated by a rapid RNA isolation method using the TRIZOL reagent (Life Technologies) according to the manufacturer's instructions and then subjected to electrophoresis through 1% formaldehyde-agarose gels, followed by transfer to Nytran nylon membranes. Blots were pre-hybridized and hybridized at 65°C, using Rapid-Hyb (Amersham) according to the manufacturer's instructions. Hybridization occurred in the presence of either an ODC cDNA probe from pODC934 or a SAMDC cDNA probe from clone H2 which encodes for mouse SAMDC. Loading of RNA samples was monitored using ethidium bromide stained ribosomal bands prior to transfer of the gels or by monitoring glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression with a GAPDH specific insert isolated from pBssK-. Densitometric analysis of appropriately exposed autoradiograms was performed using a GS-700 Imaging Densitometer (Bio-Rad Laboratories) and the Molecular Analyst software program (Bio-Rad Laboratories).

Determination of ODC and SAMDC enzyme activity

ODC and SAMDC enzyme activities were measured essentially as previously described [13, 31]. ODC enzyme activity was determined by measuring the release of [^{14}-C]-CO_2 from DL-[^{14}-C] labelled ornithine and SAMDC enzyme activity was determined by measuring the liberation of [^{14}-C]-CO_2 from S-[carboxyl-^{14}C] adenosyl-L-methionine. Radioactivity was determined by liquid scintillation spectroscopy using a LS6500 multipurpose scintillation counter (Beckman). Enzyme activity was determined and expressed as nmoles CO_2/h/mg prot. Protein content was determined using the Biorad reagent (Bio-Rad).

Determination of ornithine decarboxylase and SAMDC transcription rates

ODC and SAMDC transcription rates were determined by nuclear run on assays, essentially as described previously [32]. Nuclei were prepared according to established methods [33]. Nuclei isolated were intact and free of cellular debris as assessed by phase-contrast microscopy. Nuclear transcription activity was determined by measuring [α^{32}-P]-UTP incorporation in RNA transcripts elongated *in vitro*. ODC, SAMDC, GAPDH, and pBR322 cDNAs (pBR322 served as a negative control) were linearized appropriately. Quantitative results were obtained by densitometric scanning and are expressed with reference to the signal for GAPDH.

Assay for invasion into collagen gels

Assessment of the invasion of cells into collagen gels was done essentially as described previously [8]. Briefly, collagen gels (using rat tail collagen) were prepared by mixing collagen, double distilled water, and 5 × concentrated Dulbecco's modified Eagle's medium (Gibco-BRL) in a ratio of 5:3:2. This results in a final collagen concentration of about 1.5 mg/ml in an isotonic buffered solution. The collagen solution was added to wells in a 6-well plate and the plates were incubated in a 5% CO_2 incubator at 37°C to permit collagen polymerization to occur. Cells (10×10^4 cells) were then added in a volume of 2 ml of alpha minimal essential media containing 10% serum onto the top of the polymerized collagen. Collagen invasion was quantified by determining the number and the depth from the surface of each cell within several fields after a 48 h period. The top surface of the gel was brought into focus and the number of cells in that focal plane was determined. Cells that had invaded below the surface of the gel were brought into focus and the depth of each cell was measured using a fine adjustment knob micrometer. Cells that had invaded less than 10 μm deep were considered to be on the surface of the gel due to the size of the cells being studied. The percentage invasion relates the number of cells 10 or more micrometers below the surface to the total number of cells in several fields. The depth of invasion is the average depth to which all invading cells had migrated.

Results

ODC and SAMDC expression in K-FGF over-expressing cells

The level of ODC and SAMDC expression in exponentially growing cells was determined by Northern blot analysis and by radiochemical enzyme assay. As shown in Fig. 1, increased levels of both ODC and SAMDC mRNA levels were noted in the K-FGF over-expressing cell lines. ODC and SAMDC mRNA levels were elevated about 4.5- and 15-fold and 6.5- and 12.5-fold in the 3G and the 3G-20 cell lines, respectively, relative to the levels found in the parental dC2 cell line. These increases in both ODC and SAMDC mRNA levels were accompanied by corresponding increases in ODC and SAMDC enzyme activity. ODC enzyme activity was increased by about 6- and 13.4-fold in the 3G and the 3G-20 cell lines relative to the level of ODC enzyme activity in the dC2 parental cell line (Table 1). Similar increased levels of SAMDC enzyme activity were also noted. SAMDC enzyme activity was increased by about 6- and 6.9-fold in the 3G and the 3G-20 cell lines relative to the level of SAMDC enzyme activity found in the parental dC2 cell line (Table 1).

Mechanisms responsible for the altered expression of ODC and SAMDC mRNA levels

Alterations in mRNA expression levels may be accounted for by a number of mechanisms including alterations in the rate

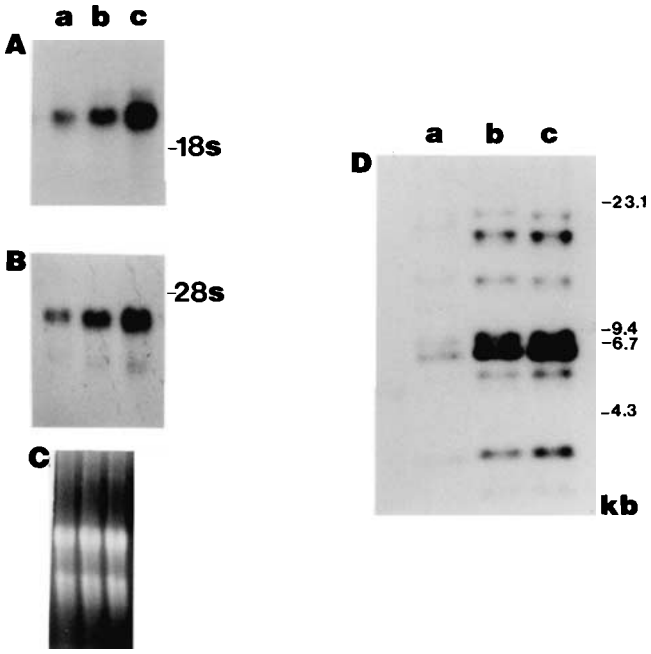

Fig. 1. Northern blot analysis of mRNA expression. (A) Northern blot analysis of ODC mRNA levels in dC2 cells (a), in 3G cells (b) and in 3G-20 cells (c). (B) Northern blot analysis of SAMDC mRNA levels as described above. (C) Ethidium bromide stained ribosomal bands are shown as a loading control. (D) Southern blot analysis of ODC DNA from dC2 cells (a), 3G cells (b) and 3G-20 cells (c). Genomic DNA (20 μg) was digested to completion with Hind III restriction endonuclease. DNA size markers are shown on the right. The autoradiograms shown in (A), (B) and (D) were exposed for 24, 24 and 48 h, respectively, at –70°C with intensifying screens.

Table 1. Ornithine decarboxylase and S-adenosylmethionine decarboxylase enzyme activity in exponentially growing K-FGF over-expressing cells

Cell line	ODC enzyme activity (nmoles CO_2/h/mg prot)	SAMDC enzyme activity (nmoles CO_2/h/g protein)
dC2	0.12 ± 0.05	0.14 ± 0.06
3G	0.72 ± 0.09	0.84 ± 0.09
3G-20	1.61 ± 0.22	0.96 ± 0.08

Results presented are from duplicate assays done in triplicate.

of gene transcription and by mechanisms of post-transcriptional regulation. Altered mRNA expression may also be accounted for by mechanisms of gene amplification. As indicated in Table 2, the relative rate of transcription of both ODC and SAMDC was increased in the 3G and in the 3G-20 cell lines. Although K-FGF over-expression-mediated elevations in ODC and in SAMDC mRNA levels may occur via changes in the transcriptional process, growth factors can also regulate gene expression post-transcriptionally through alterations in message stability [11, 13]. Therefore, the possibility that changes at the post-transcriptional level had occurred in response to K-FGF over-expression was studied. As shown in Figs 2 and 3, alterations in the half-life of both ODC and SAMDC mRNA transcripts in response to K-FGF over-expression had occurred. The half life of ODC message in dC2 cells was determined to be about 2.8 h, whereas the half-life of ODC message in the 3G and in the 3G-20 cell lines was determined to be about 6 and 14.5 h, respectively (Fig. 2). The half-life of SAMDC message in the dC2 cell line was determined to be about 3 h, whereas in the 3G-20 cell line, the half life of SAMDC mRNA transcripts was determined to be about 8 h (Fig. 3). These findings indicate that K-FGF mediated alterations in both ODC and SAMDC expression occur partly through regulation of ODC and SAMDC mRNA levels through a mechanism(s) of post-transcriptional stabilization. Additionally, an interesting part of the altered phenotype associated with K-FGF over-expression is an apparent amplification of the ODC gene in both the 3G and the 3G-20 cell lines (Fig. 1). No amplification of the SAMDC gene occurred in these cells (data not shown).

Table 2. Relative rates of ODC and SAMDC gene transcription in exponentially growing K-FGF transformants

Cell line	Relative rate of gene expression (Fold increase)[1]	
	ODC	SAMDC
3G	2.1	2.2
3G-20	2.5	3.1

[1]The fold increase is relative to the level of transcription determined to occur in the control dC2 cell line.

Collagen gel invasion by cells over-expressing K-FGF

The ability to invade collagen gels is often observed with populations of highly malignant cells [8]. Therefore, the effect of K-fgf gene expression on the invasive characteristics of cells on a physically relevant collagen substratum was determined. As shown in Fig. 4, the untransformed dC2 cell line exhibited low levels of invasion with only about 2.5% of the cells occurring below the surface of the gel after 48 h. The 3G-20 cell line was more aggressive in this assay when compared to the control dC2 cell line, with about 41% of the cells occurring below the surface of the gel post-48 h of culture (Fig. 4). These observations are in general agreement with previously reported observations with these cells [8]. Difluoromethylornithine (DFMO) is an inhibitor of ornithine decarboxylase. Interestingly, in the presence of DFMO (Sigma), (5 mM), the ability of 3G-20 cells to invade the collagen matrix was markedly reduced, with only about 16% of the 3G-20 cells occurring below the surface of the gel post-48 h of culture (Fig. 4). 3G-20 cells in the presence of DFMO (5 mM) and in the presence of putrescine (Sigma), (10 mM), were able to invade the gel with a greater ability. In this case, about 35% of the 3G-20 cells were located below the surface of the gel (Fig. 4). In the presence of exogenous putrescine (10 mM) alone, about 38% of the 3G-20 cells occurred below the surface of the gel (Fig. 4). The depth to which the K-fgf -transfected cells invaded was also determined. The dC2 cells located below the surface of the gel had invaded to an average depth of 30.5 ± 5.0 μm, whereas the 3G-20 cells had invaded to an average depth of 46.5 ± 5.5 μm. These values are comparable to previously reported observations [8]. Interestingly, 3G-20 cells cultured in the presence of putrescine (10 mM) alone were able to invade the surface of the gel with approximately the same degree of invasion. However, putrescine treated 3G-20 cells were able to penetrate the surface of the collagen gel to a greater extent than 3G-20 cells not treated with putrescine. Putrescine treated 3G-20 cells were able to invade the gel to an average depth of 56.5 ± 3.5 μm. (Putrescine treatment and DFMO treatment had little to no appreciable effect on either the ability of dC2 cells to invade or on the extent to which these cells were able to penetrate the gel).

Discussion

Fibroblast growth factors have many physiological roles including having the ability to contribute to the process of tumour formation and malignancy [2, 3, 8]. This study's analysis of the relationship between altered expression of ODC and SAMDC, the two key regulatory activities of polyamine biosynthesis, and the metastatic potential of NIH-3T3 fibroblasts transfected with K-fgf sequences is in support of this con-

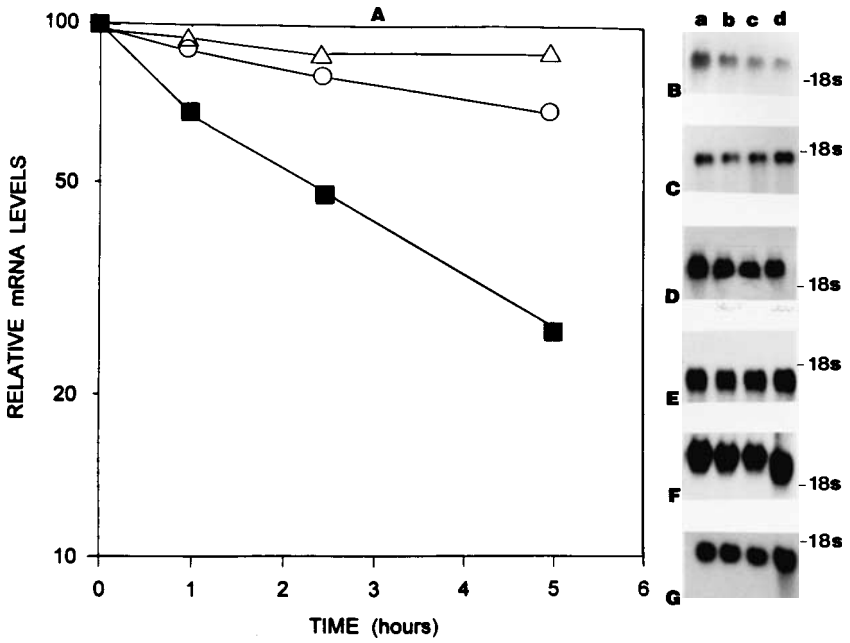

Fig. 2. Stability of ODC mRNA. (A) Stability of ODC mRNA in dC2 cells (■), in 3G cells (O), and in 3G -20 cells (Δ). Exponentially growing cells were subsequently treated with 5,6 dichloro-1-beta-D-ribofuranosylbenzimidazole, (DRB), (80 μM), for pre-determined times. Total RNA was isolated at 1, 2.5, and 5 h after treatment with DRB and subjected to Northern blot analysis as described in 'Materials and methods'. The relative levels of ODC mRNA were evaluated by densitometric evaluation of autoradiograms exposed in the linear range for each set of samples and the values normalized to GAPDH levels which were used as controls. Representative results are presented in (A). Representative Northern blot analyses are also presented. (B) Northern blot analysis of ODC mRNA expression levels in dC2 cells (control cells) (a) , in dC2 cells following exposure to DRB for 1 h (b) , for 2.5 h (c) and for 5 h (d). (C) GAPDH mRNA levels are shown as a loading control. (D) ODC mRNA expression levels in 3G cells as described in (B). (E) GAPDH mRNA levels are shown as a loading control. (F) ODC mRNA expression levels in 3G-20 cells as described in (B). (G) GAPDH mRNA expression levels are shown as a loading control.

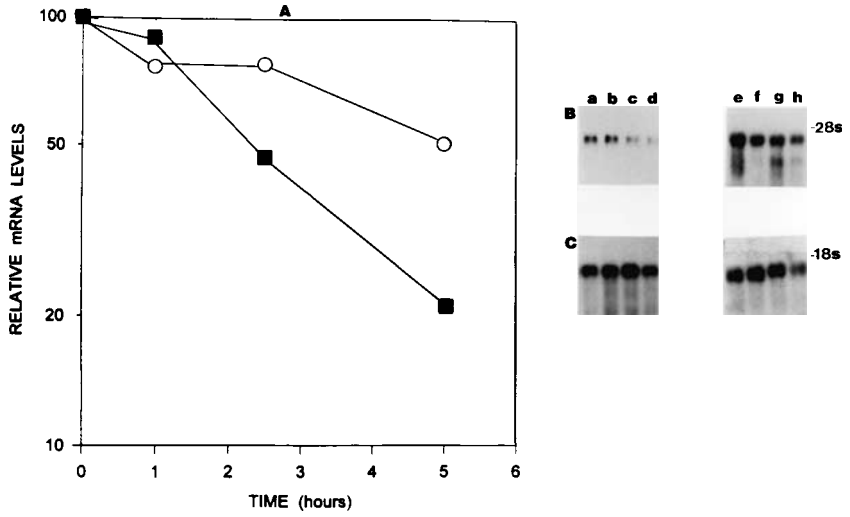

Fig. 3. Stability of SAMDC mRNA. (A) Stability of SAMDC mRNA in dC2 cells (■) and in 3G-20 cells (O). Exponentially growing cells were subsequently treated with DRB, (80 μM), for pre-determined times. Total RNA was isolated at 1, 2.5, and 5 h after treatment with DRB and subjected to Northern blot analysis as described in 'Materials and methods'. The relative levels of SAMDC mRNA were evaluated by densitometric evaluation of autoradiograms exposed in the linear range for each set of samples and the values normalized to GAPDH levels which were used as controls. Representative results are shown in (A). Representative Northern blot analysis is also presented. (B) Northern blot analysis of SAMDC mRNA levels in dC2 cells (control cells) (a), and in dC2 cells following exposure to DRB for 1 h (b), for 2.5 h (c) and 5 h (d), and in 3G-20 cells (control cells) (e), and in 3G-20 cells following exposure to DRB for 1 h (f), for 2.5 h (g) and 5 h (h). (C) GAPDH mRNA expression levels are indicated as a loading control.

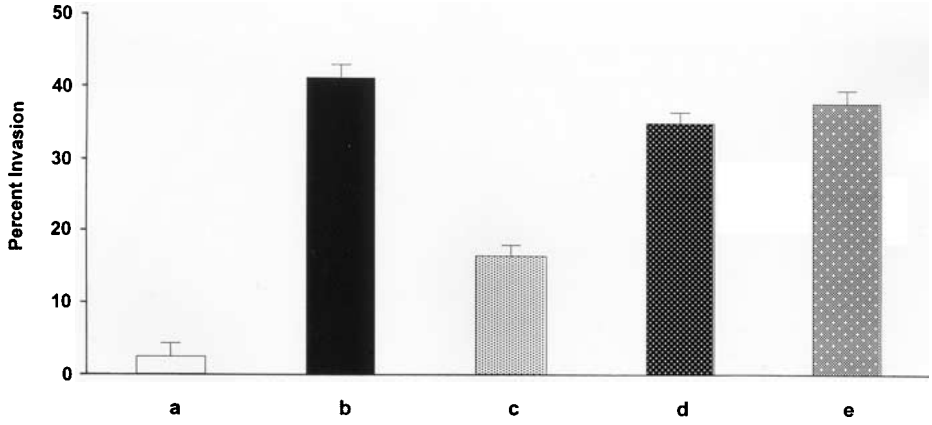

Fig. 4. Invasion of collagen gels. The percentage of cells below the surface of the gel relative to the total cell number observed in three to four microscopic fields (average of 2 trials) is shown for dC2 cells (a), and for 3G-20 cells (b), and for 3G-20 cells grown in the presence of DFMO (5 mM) (c), and for 3G-20 cells grown in the presence of DFMO (5 mM) and in the presence of putrescine (10 mM) (d), and for 3G-20 cells grown in the presence of added putrescine (10 mM) (e).

cept since all the highly malignant cells examined in this study possessed alterations in ODC and in SAMDC expression. These observations suggest that one of the parameters, which is altered as a direct consequence of K-fgf over-expression and which contributes to malignant progression, is altered expression of ODC and SAMDC. In this regard, this is the first report to demonstrate the potential of K-FGF as a critical and potent regulator of ODC and SAMDC expression. Interestingly, the alterations which occurred correlated with the malignant potential of the K-FGF over-expressing cells.

ODC and SAMDC over-expression in these K-FGF over-expressing cells was the consequence of a number of different mechanisms including transcriptional and post-transcriptional events. Amplification of the ODC gene also contributes to the resulting cellular phenotype. The expression of K-fgf in these cell lines has been previously determined [7]. These previous studies showed that the proto-oncogene K-fgf was amplified in these NIH-3T3 transfected cells and revealed that the K-fgf transfected and gene amplified cell lines were highly aggressive [7, 8]. Amplification of the K-fgf gene occurred only in the 3T3.3G series of cells, and this was coupled with the production of detectable levels of K-fgf mRNA and alterations in K-fgf protein expression [7]. The increased stability of both the ODC and the SAMDC mRNA transcripts in the K-FGF over-expressing cells most probably represents a part of the altered growth regulatory programs found in these cells and as such may impart upon these cells a selective growth advantage which contributes to their increased aggressive behaviour.

Altered fibroblast growth factors may play a fundamental role in malignant progression by affecting the genetic stability of cells and contributing to a mechanism for generating variants for malignant selection [34]. One of the important characteristics of malignant cells that distinguishes them from normal cells is their ability to amplify DNA at relatively high rates [34]. Previously, it has been demonstrated that altered expression of bFGF in NIH-3T3 cells markedly increases the resistance of these cells to N-(phosphonacetyl)-L-aspartate (PALA) through a mechanism that is mediated by CAD gene amplification [34]. This finding suggested an important new role for bFGF over-expression in the process of tumour progression. The aberrant expression of bFGF can contribute to malignant progression by affecting the genetic stability of cells thereby providing a mechanism for generating variants that are essential for malignant selection [34]. K-FGF over-expression may also be functioning in a similar manner. K-FGF over-expression in cells may result in the selection of variants harbouring alterations which would facilitate malignant progression. Alterations in ODC and in SAMDC expression and regulation which occur in K-FGF over-expressing cells may afford these cells a more aggressive phenotype or at least endow these cells with alterations which enhance their abilities to form tumours.

Although the transforming potential of K-fgf and its ability to regulate cell proliferation has been documented [2], this is the first report to demonstrate the potential of this growth factor as an important regulator of the activities associated with polyamine biosynthesis. The potential of K-fgf as a potent regulator of cell motility has previously been demonstrated, as has its ability to increase the invasive potential of cells [8]. In this regard, this study is the first to suggest that a key factor associated with the ability of K-FGF over-expressing cells to invade is the increased expression of ODC and by extension, alterations in cellular polyamine levels. Not only was ODC expression important in order for invasion to occur, but, it would seem that putrescine is also an important contributing factor. Other studies have implicated a role for putrescine in cellular invasion [29]. In rat ascites

hepatoma cells, the DFMO induced decreases in invasive capacity and putrescine levels were almost completely reversed by the addition of putrescine to the medium during pre-treatment with DFMO or invasion assay, but were not affected by exogenous spermidine or spermine [29]. The invasive capacity of rat ascites hepatoma cells was, therefore, putrescine dependent [29]. This also hold true for the 3G-20 cells which over-express K-fgf. Interestingly, putrescine addition alone apparently does not enhance the degree of invasive capacity of these cells. However, putrescine addition does apparently increase the depth to which these cells can invade the collagen matrix. This may represent an important aspect of the role of putrescine to tumour biology. By increasing the depth to which transformed cells can invade may ultimately enhance their ability to progress and metastasize.

It is important to note that alterations in ODC and in SAMDC expression probably represent only one aspect of the transformation phenotype associated with K-fgf over-expression since over-expression of ODC and SAMDC on their own is presumably not sufficient on their own to induce metastasis. As such, this would suggest that other biological modifications are also necessary for cellular metastasis to occur. In this regard, studies examining the expression of matrix metalloproteinases and other proteases in these NIH-3T3 cells which overexpress K-FGF indicate marked increased expression of a number of proteases including matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) (unpublished observations). Correlations between altered matrix metalloproteinases' expression and malignant potential of these cells exist and correlations between the expression of MMP-2 and MMP-9 and the invasion ability of the K-FGF over-expressing cells also exist (unpublished observations). These observations suggest that in addition to alterations in ODC and in SAMDC expression, alterations in proteases are also part of the altered growth regulatory program associated with cellular transformation and malignant potential.

Alterations in ODC and in SAMDC have been suggested to be important contributors to cellular transformation events including the development of tumours and the onset of malignant progression [26, 28, 35, 36]. The signalling events in the transduction mechanisms initiated by fibroblast growth factors, including K-FGF, to their respective receptors, presumably activate cellular proliferation and other events important in determining the cellular phenotype which is ultimately expressed. This is probably appropriate to the events of cellular transformation and the generation of the metastatic cascade. This study suggests that one of these critical determining events involves alterations in the expression of ODC and SAMDC , the key regulators of polyamine biosynthesis.

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