Inhibition of transforming growth factor-β-activated kinase-1 blocks cancer cell adhesion, invasion, and metastasis

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BACKGROUND: Tumour cell metastasis involves cell adhesion and invasion, processes that depend on signal transduction, which can be influenced by the tumour microenvironment. N-6 polyunsaturated fatty acids, found both in the diet and in response to inflammatory responses, are important components of this microenvironment.

METHODS: We used short hairpin RNA (shRNA) knockdown of TGF-β-activated kinase-1 (TAK1) in human tumour cells to examine its involvement in fatty acid-stimulated cell adhesion and invasion in vitro. An in vivo model of metastasis was developed in which cells, stably expressing firefly luciferase and either a control shRNA or a TAK1-specific shRNA, were injected into the mammary fat pads of mice fed diets, rich in n-6 polyunsaturated fatty acids. Tumour growth and spontaneous metastasis were monitored with in vivo imaging of bioluminescence.

RESULTS: Arachidonic acid activated TAK1 and downstream kinases in MDA-MB-435 breast cancer cells and led to increased adhesion and invasion. Knockdown of TAK1 blocked this activation and inhibited both cell adhesion and invasion in vitro. Tumour growth at the site of injection was not affected by TAK1 knockdown, but both the incidence and extent of metastasis to the lung were significantly reduced in mice injected with TAK1 knockdown cells compared with mice carrying control tumour cells.

CONCLUSION: These data demonstrate the importance of TAK1 signalling in tumour metastasis in vivo and suggest an opportunity for antimetastatic therapies.

Keywords: adhesion; invasion; metastasis; fatty acid; TGF-β-activated kinase-1; breast cancer

Tumour cell metastasis is a complex, multi-step process involving cell adhesion, invasion, and migration on extracellular matrix (ECM). During this process, cell signalling is critical for the interaction of tumour cells with other cells, the ECM, and components within tumour microenvironments (Talmadge and Fidler, 2010). We have focussed on defining and characterising pathways (Paine et al, 2000; Palmantier et al, 2001) that are activated by polyunsaturated fatty acids found both in typical Western diets (Rose and Connolly, 1997) and in response to inflammatory signals (Honn et al, 1992). We hypothesise that one critical pathway involves TGF-β-activated kinase-1 (TAK1) (Nony et al, 2005).

TGF-β-activated kinase-1 is a member of the mitogen-activated protein kinase (MAPK) family, and has been implicated in various signalling pathways (Yamaguchi et al, 1995; Ninomiya-Tsuji et al, 1999). The downstream targets of TAK1 include MKK and IKK, which in turn can activate p38 MAPK, JNK, or NF-κB signalling pathways (Landstrom, 2010). Thus, TAK1 regulates a number of biological processes including immune responses, stress responses, and inflammation (Landstrom, 2010).

Tumour cells can be strongly affected by components of the extracellular microenvironment (Talmadge and Fidler, 2010), including the n-6 polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA) and its precursor linoleic acid. Both fatty acids are consumed in substantial amounts in Western diets. In tumour cells, PUFAs have been shown to stimulate an adhesive and invasive phenotype in breast cancer cells in vitro and to promote tumour growth and metastasis in animal models (Rose et al, 1991; Connolly and Rose, 1993; Palmantier et al, 1996). Fatty acids induce various signal transduction pathways in MDA-MB-435 cells, a highly metastatic human breast cancer cell line frequently used in models of breast cancer (Hurst et al, 2009). This cell line is particularly useful for the study of metastasis, given its preference for growth in a mammary environment (Bao et al, 1994), and displays an aggressive, metastatic phenotype accompanied by the expression of both mammary marker proteins and...
melanocyte-related genes, similar to patterns observed in freshly resected, human breast tumours (Montel et al, 2009). Activation of these cells with fatty acids in vitro leads to β1 integrin activation and increased cell adhesion to collagen IV (Palmantier et al, 2001). Arachidonic acid activates a p38 MAPK pathway that is required for adhesion to collagen IV (Paine et al, 2000; Garcia et al, 2009). Thus, in vitro, these tumour cells take on many of the characteristics of highly metastatic cells, consistent with the increased metastasis seen in animal models in which mice are fed high levels of dietary n-6 PUFAs (Rose and Connolly, 1997).

Preliminary evidence from our laboratory suggested that TAK1 may have a role in AA-induced p38 activation (Nony et al, 2005). Herein, we investigated the role of TAK1 in cancer cell adhesion and invasion in vitro, and in tumour growth and metastasis in vivo.

MATERIALS AND METHODS

Cell culture and treatment conditions

MDA-MB-435 cells were from Dr Janet Price at the MD Anderson Cancer Centre (Houston, TX, USA) (Price et al, 1990) and cultured as described. SK-Mel cells were obtained from the ATCC (Manassas, VA, USA) and cultured as recommended. Cell treatments were performed as described (Ray et al, 2010).

Immunoblotting

Cell lysates were prepared and immunoblots were performed as described (Ray et al, 2010). Membranes were immunoblotted for phospho-TAK1 (Thr187) or phospho-p38 (Thr180/182) (Cell Signaling Technology Inc., Danvers, MA, USA), and probed for TAK1, p38, GAPDH, or HA-tag (Millipore, Temecula, CA, USA). Densitometric analysis was performed using Gene Tools software from Syngene (Frederick, MD, USA).

Generation of stable cell lines

Short hairpin RNA (shRNA) sequences against TAK1 or a control sequence were cloned into the pSilencer 3.1-H1 hygro vector from Ambion Inc. (Austin, TX, USA) according to the manufacturer’s protocol. The following hairpin RNA sequences were designed:

TAK1 target #1 (5’-GATCGAGAAGTCTGAGGGATGCTTAAGAAGGAC-TAACACATCAGGATTCTTTTTTGGAAA-3’); TAK1 target #2 (5’-GATCCGAGAACCTTGGCTTACAAAAGAGATTTTGAGGAATGAGAATGTGTTTTTGGAAA-3’); Control (5’-GATCCGAGAACCTTGGCTTACAAAAGAGATTTTGAGGAATGAGAATGTGTTTTTGGAAA-3’).

Cells were transfected with 0.7 μg DNA using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN, USA). Hygromycin- (Invitrogen) resistant colonies were selected. A two-tailed Student t-test was performed for adhesion and invasion assays, the Tukey–Kramer test was used for analysing tumour size, and the log-rank test was used for the metastasis data. A P-value less than 0.05 was considered statistically significant. All analyses were performed using statistical software package, SAS version 9.01 (SAS Institute Inc., Cary, NC, USA).

Transient transfections

Transient TAK1 transfections to overexpress human TAK1 were performed using a pCMV-HA-TAK1 vector, kindly provided by Jun Ninomiya-Tsuji (North Carolina State University, Raleigh, NC, USA). Cells were transfected with pCMV-HA-TAK1 or an empty vector control (mock) using FUGENE 6 reagent as described above. After 24 h, the transiently transfected cells were exposed to vehicle or AA. Immunoblots were performed to confirm HA-TAK1 protein expression.

Adhesion and invasion assays

Adhesion assays were performed as described previously (Garcia et al, 2009).

Invasion of cells through Matrigel- (BD Biosciences, Franklin Lakes, NJ, USA) (1:25 dilution) coated nucleopore membranes (8 μm pores) (Fisher Scientific, Pittsburg, PA, USA) was performed in Boyden chambers (Neuro Probe, Gaithersburg, MD, USA) for 1 h at 37°C. Ten percent FBS was placed in the bottom chamber and 2.5 × 104 cells were placed in the top chamber with 1% FBS medium for 24 h. The invaded cells were stained with Diff-Quik (Dade Behring Holdings Inc., Deerfield, IL, USA) and the cells were counted in four fields per membrane on triplicate membranes.

Spontaneous metastasis and bioluminescent imaging

All animal experiments were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee and followed the NIH guidelines for the care and use of laboratory animals. The mice were maintained on the NIH-31 diet containing 4.6% (w/w) linoleic acid, 4.7% n-6 PUFAs, and 5.2% total PUFAs (Shen et al, 2007). A total of 1 million control shRNA- or TAK1 #1 shRNA luciferase-expressing cells were injected into the right inguinal mammary fat pad of 6-week old female nude mice (NU/Nu/B+C from Charles River, Raleigh, NC, USA) by close injection using a 25-gauge needle. Tumour development was monitored weekly by bioluminescent imaging using the Spectrum in vivo imaging system following the manufacturer’s protocol (Caliper Life Sciences, Hopkinton, MA, USA) and quantified using the LivingImage software (Caliper). Tumour size was monitored and tumours were surgically resected when they reached a length of 10–15 mm. Tumour volumes were calculated using the formula for an oblate spheroid: 4/3πL×W, where L is the length and W is the width (Matsuoka et al, 2010). Mice were monitored for an additional 8 weeks beyond tumour resection. For ex vivo imaging of organs, mice were injected with d-luciferin 10 min before euthanising with Fatal-Plus Solution (Vortech Pharmaceuticals Ltd, Deerborn, MI, USA). This experiment was repeated twice with a total of 23 mice in the control shRNA group and 21 in the TAK1 shRNA group. The bioluminescence images presented herein were normalised to total flux (Radiance) and the images in each figure are presented on the same luminescence scale.

Pathological analysis

The lungs were step sectioned at 250 μm increments with 4 sections taken at each step. The sections were stained with haematoxylin and eosin (H&E) and examined for tumour burden. Slides were digitally scanned on the Scanscope XT (Aperio Technologies, Vista, CA, USA). Regions of tumour cells were annotated in the left lobe of the lung. Image analysis was performed using the Aperio Positive Pixel Count Algorithm. The area of the total number of pixels counted was generated for the annotated regions. For the left lobe area, the algorithm was repeated and the area recorded. To determine the per cent tumour burden, the area of positive stained pixels for the annotated regions was divided by the total area of positive pixels counted. Image analysis was performed on three slides from each lung (at intervals of one-quarter, one-half, and three-quarters through the lung) and the median value was calculated.

Statistical analysis

A two-tailed Student t-test was performed for adhesion and invasion assays, the Tukey–Kramer test was used for analysing tumour size, and the log-rank test was used for the metastasis data. A P-value less than 0.05 was considered statistically significant. All analyses were performed using statistical software package, SAS version 9.01 (SAS Institute Inc., Cary, NC, USA).
RESULTS

TGF-β-activated kinase-1 is rapidly phosphorylated

Previously radiolabelling assays showed that AA induced phosphorylation of a protein similar in electrophoretic mobility to TAK1 (Nony et al, 2005). To confirm that TAK1 is phosphorylated, we performed a time-course analysis of TAK1 auto-phosphorylation using a specific phospho-TAK1 antibody. Phosphorylation of TAK1 T187 was detected as early as 2 min after AA treatment (Figure 1), and maintained for at least 10 min. Phosphorylation of MAPK p38 paralleled TAK1 phosphorylation (Figure 1), but consistent with previous studies, seems to have a slightly delayed maximal response, consistent with TAK1 being an early signalling molecule, and p38 activation occurring later in the pathway (Paine et al, 2000; Nony et al, 2005). These experiments were also performed on SK-Mel-3, a human melanoma cell line, and AA induced phosphorylation of TAK1 and p38 in these cells (Supplemental Figure 1).

TGF-β-activated kinase-1 is required for p38 MAPK activation, adhesion and invasion

MDA-MB-435 cells stably expressing either a control shRNA or one of two different TAK1 shRNAs were generated. TGF-β-activated kinase-1 protein was reduced by 77% and 85% in the two TAK1 shRNA clones compared with control cells (Figure 2) and cell proliferation did not change (Supplemental Figure 2). As shown in Figure 2A, AA induced p38 phosphorylation in the control shRNA expressing cells, but was unable to induce p38 phosphorylation in TAK1 knockdown cells. A rescue experiment was performed in TAK1 shRNA cells transiently overexpressing TAK1. Overexpression of TAK1 allowed the cells to regain the ability to phosphorylate p38 in response to AA (Figure 2B).

Next, the parental MDA-MB-435, control shRNA, and the two TAK1 shRNA cell lines were tested for adhesion to collagen IV and invasion through Matrigel. Control shRNA and parental cells had similar adhesion responses, with AA inducing a three-fold induction of cell adhesion as compared with the control (Figure 3). In contrast, the TAK1 knockdown cells were unable to increase their adhesion to collagen IV in response to AA treatment, demonstrating the requirement for TAK1. Control shRNA cells and parental MDA-MB-435 cells demonstrated a significant increase in invasion when treated with AA when compared with the vehicle-treated control (Figure 4A). However, shRNA knock-down of TAK1 completely blocked the AA-induced invasion in both TAK1 shRNA cell lines (Figure 4B).

Figure 1 TGF-β-activated kinase-1 is phosphorylated upon AA exposure. Immunoblots were performed for phospho-TAK1-Thr187 or phospho-p38 and re-probed for total TAK1, total p38, and GAPDH. Results are representative of three experiments.

Figure 2 TGF-β-activated kinase-1 is required for p38 activation. (A) Cells stably expressing TAK1 shRNA #1, TAK1 shRNA #2, or a control shRNA were exposed to ethanol (−) or AA (+) for 5 min, immunobloted for phospho-p38 and re-probed for total p38 and total TAK1. The numbers are the ratio of TAK1 protein compared with the TAK1 in the control shRNA cells (set to 1.0). (B) The TAK1 shRNA #1 cell line was transiently transfected with HA-TAK1 for 24 h, exposed to ethanol (−) or AA (+) for 5 min, and analysed for phospho-p38, total p38 and HA-TAK1. Results are representative of 3 experiments.

Figure 3 TGF-β-activated kinase-1 is required for AA-induced adhesion to collagen IV. Cells were exposed to ethanol or AA for 45 min. The results are presented as the per cent adhesion to collagen IV using adhesion to poly-D-lysine as 100% adhesion. Shown is one representative of 3 experiments. Error bars represent one standard deviation from the mean. *P<0.05 compared with AA-treated MDA-MB-435 and control shRNA-containing cells.

TGF-β-activated kinase-1 shRNA expressing cells form tumours

We investigated the effect of TAK1 knockdown on the ability of the cells to form tumours in an orthotopic model in mice continuously fed a diet containing 4.6% linoleic acid. Exposure of the animals to the dietary fatty acids continued throughout the experiment.

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A control shRNA cell line and a TAK1 shRNA cell line expressing luciferase were (Supplemental Figure 3) injected into the mammary fat pad and monitored for bioluminescence and tumour growth. Both cell lines formed tumours at the site of injection (Figure 5A). The TAK1 shRNA tumours displayed a slightly higher luminescence than the control shRNA tumours (Supplemental Figure 4A), which could be due to either larger size or reduced depth of the tumour. On the basis of caliper measurements, the TAK1 shRNA tumours appeared slightly larger than the control shRNA tumours (Figure 5B). This suggested that the TAK1 shRNA tumours became established slightly faster than the control shRNA tumours, but the control tumours grew quickly after 5 weeks, and by 7 weeks, were not statistically different in size from the TAK1 shRNA tumours. These findings suggest that any differences in metastasis are not likely the result of differences in tumour size or initial tumour growth.

We used tumour size, instead of time following injection, as the criterion for resection, because tumour size is strongly dependent on angiogenesis, which is required for growth and which provides a route for tumour cells to escape the original site (i.e., haematogenous metastasis) (Folkman, 2002; Weinberg, 2007). As most tumours spread by haematogenous metastasis, allowing the tumours to reach a similar size should allow for a more equivalent opportunity for metastasis. The TAK1 shRNA tumours were removed at week 8 and the control shRNA tumours were removed at week 9 following injection, at which time the tumours had equivalent volumes (Figure 5C).

After primary tumour resection, the mice were imaged weekly for metastatic tumours. In the control group, lung metastases were detectable as early as 1-week post-resection (Figure 6A). There were distinct luminescent foci that appeared to be lung tumours. The per cent of control mice with metastasis increased steadily over the 7 weeks (Figure 6B). As depicted in Figure 6A, a few of the TAK1 shRNA mice developed metastases, but many of the mice were negative (e.g., mouse #4). In fact, as seen in Figure 6B, only 38% of mice in the TAK1 shRNA group had metastases compared with 83% for the control shRNA group at 7 weeks post resection. Additionally, the number of control shRNA mice that developed metastases steadily increased over time. However, the number of TAK1 shRNA mice with metastases only increased slightly over the 7-week time course. Overall, the number of mice that developed metastases over time was significantly higher in the control group compared with the TAK1 shRNA group ($P = 0.004$ by log-rank test). The trend over time revealed that the TAK1 shRNA group had a significantly lower luminescence (Supplemental Figure 4A).

Furthermore, more animals in the control group displayed evidence of metastasis as early as 2 weeks after the tumour resection. Thus, it is unlikely that the 1-week difference in time that the animals carried the primary tumour resulted in the striking difference in metastasis observed between the two groups.

**Ex vivo imaging and pathological evaluation of lung tumour burden**

At 8 weeks post-primary tumour resection, tissues were imaged ex vivo (Figure 7A). These images confirmed that the tumours were in the lung. In total, from 2 separate experiments, 20 out of 23 mice in the control shRNA group developed lung metastasis (Figure 7B). In contrast, only 10 out of 21 mice in the TAK1 shRNA group developed lung metastasis. This decrease in the incidence of metastasis was highly significant ($P = 0.002$). Quantification of the lung bioluminescence revealed that although there appeared to be a slight decrease in luminescence in the TAK1 shRNA group compared with the control shRNA group, this difference was not statistically significant (Supplemental Figure 4C). No metastases were detected in the liver, spleen, bone, or brain (data not shown).

Pathological analysis of the lungs confirmed the lung bioluminescence results (data not shown). On the basis of H&E lung sections, the per cent tumour burden in the TAK1 shRNA group was...
vs 23.4 ± 20.4, respectively; \( P = 0.03 \) (Figure 7C).

**DISCUSSION**

This study demonstrates that TAK1 is a critical component for the induction of increased adhesion, invasion, and metastasis. Most importantly, this is the first report demonstrating that TAK1 knockdown in an orthotopic model of spontaneous breast cancer metastasis results in a significant reduction in metastasis frequency and a striking decrease in lung tumour burden. In the present study, we found that the MDA-MB-435 control shRNA cells formed tumours in the mammary fat pad and were highly metastatic to the lung in animals on the normal NIH-31 rodent diet. These numbers are comparable to what is described for the MDA-MB-435 cells (Price et al., 1990). We did not detect a higher rate of metastasis in either the control or TAK1 shRNA tumour-bearing animals when they were maintained on a 12% linoleic acid diet (data not shown). This result was not surprising because we observed such a high metastatic rate in the control animals on the diet containing 4.6% linoleic acid. Therefore, the findings from our metastasis experiments are more broadly applicable in that the role of TAK1 is not likely limited to metastasis induced by high levels of fatty acids but is important in pro-metastatic signalling pathways initiated under relatively normal dietary conditions.

Interestingly, the TAK1 shRNA cells established as a tumour in vivo slightly faster than the control shRNA cells, even though, in vitro, there was no difference in growth between these two cell lines. However, we observed that the control shRNA tumours, although slower to establish initial growth, rapidly increased in size once the tumours were of palpable size. Even though, this initial difference in tumour growth was observed, the TAK1 shRNA tumours were significantly less likely to metastasise than the control shRNA tumours. Any number of factors could have influenced the initial growth of the tumour cells in vivo that would not have been present in vitro. It is possible that other pathways were affected by TAK1 knockdown that could have resulted in a different response of these cells when exposed to the extracellular environment of the mammary fat pad that allowed them to establish into a tumour faster than the control shRNA cells. This finding is in contrast to that reported in a different cell line in which a dominant negative TAK1 construct inhibited tumour growth in vivo (Safina et al., 2008). Thus, although inhibition of tumour growth may also block metastasis, our studies reveal that TAK1 is having an important role in the successful metastasis of breast tumour cells even in the presence of normal, or slightly enhanced, growth in the mammary fat pads, suggesting that inhibition of TAK1 through small molecules or dietary changes may be able to block metastasis specifically.

TGF-\( \beta \)-activated kinase-1 is an important signalling molecule that activates numerous signal transduction pathways, many of which are associated with cancer. For example, TAK1 is a key upstream kinase in the NF-kB pathway, which promotes cell survival and proliferation and which has been the focus of treatment intervention for many types of cancers. We previously showed that lysine-63-linked ubiquitination, a pathway that involves activation of signalling but does not lead to proteasomal degradation, was required for AA to induce cell adhesion (Ray et al., 2010). In contrast, the lysine-48-linked ubiquitin-mediated degradation pathway was not required for AA-mediated cell
adhesion (Ray et al., 2010), suggesting that NF-κB, which requires lysine-48 ubiquitination, does not participate in fatty acid-induced signal transduction. We do not currently understand the mechanism by which AA exposure leads to TAK1 activation, although we have examined a number of possibilities. For example, a combination of dominant negative and siRNA experiments have shown that at least TRAF 2, 5, and 6 are not responsible for activation of TAK1 (data not shown). Furthermore, both TAB1 and TAB2 are associated with TAK1 in these cells both in the presence and absence of AA (data not shown). We have previously shown that inhibition of metabolism of AA to 15S-HETE completely blocks cell adhesion and p38 phosphorylation (Palmantier et al., 1996; Nony et al., 2005); thus, interactions between this metabolite and specific kinases or their binding partners would provide interesting targets for inhibiting these processes and is consistent with previous studies of the importance of HETEs in tumour cell behaviour (Szekeres et al., 2002). We are currently investigating the biochemical interactions of 15S-HETE with various signalling proteins to determine whether this molecule is directly responsible for kinase activation.

Figure 6  Mice with TAK1 shRNA expressing tumours are less likely to develop lung metastases. (A) Bioluminescence images taken at weeks 1–5 after tumour removal. Shown here are 2 representative mice from both groups. (B) The per cent of metastasis-free mice were plotted in a Kaplan–Meier analysis. Results are combined from 2 experiments. n = 23 for control shRNA group; n = 21 for TAK1 shRNA group. The log-rank test was performed and indicated a highly significant difference between the two groups over the time course with a P-value of 0.0044.
Figure 7  Ex vivo imaging and pathological evaluation indicates lung tumour burden. (A) Ex vivo imaging was performed at autopsy, 8 weeks after primary tumour removal. Shown here are the lungs from the same mice shown in Figure 6A. (B) The number of mice with positive lung bioluminescent signal upon ex vivo imaging was graphed. Results are combined from two independent experiments. n = 23 for control shRNA; n = 21 for the TAK1 shRNA. *P = 0.002 for TAK1 shRNA vs control shRNA, using asymptotic standard normal test. (C) Lung sections were stained with H&E. The per cent tumour burden was calculated and is shown as the mean of the median tumour burden from each animal (n = 6 for control shRNA and n = 3 for TAK1 shRNA). Representative lung sections are shown at × 200 magnification, the bars indicate 1 mm. The arrows indicate tumour cells. *P = 0.03 for TAK1 shRNA vs control shRNA by a two-tailed Mann–Whitney test.

Our results demonstrate that TAK1 expression in these tumour cells is critical for key steps in tumour cell metastasis; for example, TAK1 knockdown is associated with a decrease in the ability of the tumour cells to invade through and attach to key ECM proteins. These results provide a link between the effects of the loss of TAK1 in the tumour cells in vitro, and a clear demonstration of both fewer animals developing metastases and reduced metastatic burden in the lung. On the basis of the in vitro assays, these tumour cells lose their ability to adhere to and invade the ECM in response to specific external stimuli, in this case, AA that are commonly found in the tumour microenvironment. Thus, these tumour cells apparently require TAK1 to effectively escape the primary tumour and successfully colonise a secondary site. All together, these findings demonstrate that TAK1 is an important pro-metastatic signal transducer and represents a potential target for anti-metastasis therapy.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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