EUROPEAN JOURNAL OF CANCER 43 (2007) 202–209

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Tumour suppressor function of RNase L in a mouse model

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ARTICLE INFO

Article history:
Received 30 May 2006
Received in revised form
15 August 2006
Accepted 31 August 2006
Available online 18 October 2006

Keywords:
RNase L
Interferon
Fibrosarcoma
Bone marrow
GM-CSF

ABSTRACT

RNase L is one of the key enzymes involved in the molecular mechanisms of interferon (IFN) actions. Upon binding with its activator, 5′-phosphorylated, 2′-5′ oligoadenylates (2-5A), RNase L plays an important role in the antiviral and anti-proliferative functions of IFN, and exerts proapoptotic activity independent of IFN. In this study, we have found that RNase L retards proliferation in an IFN-dependent and independent fashion. To directly measure the effect of RNase L on tumour growth in the absence of other IFN-induced proteins, human RNase L cDNA was stably expressed in P-57 cells, an aggressive mouse fibrosarcoma cell line. Three clonal cell lines were isolated in which the overexpression of RNase L was 15–20-fold of the endogenous level. Groups of five nude mice were injected subcutaneously with either the human RNase L overexpressing clones (P-RL) or control cells transfected with an empty vector (P-Vec). Tumour growth by the two cell lines was monitored by measuring tumour volumes. In the P-RL group, tumour formation was significantly delayed and the tumours grew much slower compared to the control group. Morphologically, the P-RL tumour appeared to have more polygonal cells and increased single cell tumour necrosis. Interestingly, P-RL tumours eventually started to grow. Further analysis revealed, however, that these tumours no longer expressed ectopic RNase L. Our findings suggest that RNase L plays a critical role in the inhibition of fibrosarcoma growth in nude mice.

1. Introduction

Interferons (IFNs) are a family of cytokines participating in innate immunity against a wide range of viruses and other microbial pathogens.[1] IFNs also have anti-tumour activities due to their anti-proliferative, immunoregulatory and apoptotic properties.[2] The effects of IFNs are largely mediated through proteins encoded by IFN-stimulated genes (ISGs). One well-studied ISG is RNase L, which is one of the key enzymes in the IFN-induced 2-5A system.[3] The 2-5A system consists of two types of enzymes: 2-5A synthetases and RNase L.[4] IFNs induce a family of 2-5A synthetase genes. The 2-5A synthetases require double-stranded RNA (dsRNA) for their activities. After activation by dsRNA which is frequently produced during viral infection, 2-5A synthetases convert ATP molecules to pyrophosphate (ppi) and a series of unique, 5′-phosphorylated, 2′-5′ linked oligoadenylates known as 2-5A with the general formula ppp(A2′p5′)*n*A ($n \geqslant 2$). 2-5A binds RNase L with high affinity, converting it from its inactive, monomeric state to a potent dimeric endoribonuclease, resulting in degradation of single-stranded viral and cellular RNAs. The 2-5A system mediates host defence against certain types of viruses. The

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2006.08.024

overexpression of RNase L in NIH 3T3 cells markedly enhances the antiviral function of IFN, whereas the dominant negative RNase L suppresses the antiviral activity of IFN in SVT2 cells.[5,6] Mice containing homozygous disruption of the RNase L gene succumbs to encephalomyocarditis (EMCV) infection more rapidly than infected wild type mice.[7] A broad range of viruses including HIV-1, vaccinia virus, human parainfluenza virus-3, vesicular stomatitis virus, and EMCV have shown to be inhibited in RNase L overexpressing cell lines.[8]

RNase L has been linked to apoptosis in response to viral and non-viral agents. RNase L null mice show enlarged thymus glands at early ages, suggesting that RNase L may be involved in T-cell development. *In situ* assays for DNA fragmentation on tissue sections from both the thymus and spleen reveal a reduction in apoptosis in the untreated RNase $L^{-/-}$ mice compared to the cognate wild type mice.[7] The direct activation of RNase L by introducing 2-5A into intact cells leads to apoptosis, whereas a dominant-negative RNase L decreases the numbers of apoptotic cells generated by poliovirus infection, IFN and poly (I):poly (C) as well as staurosporine treatments.[9–11] A recent study revealed that RNase L mediates virus-induced apoptosis through activating c-Jun NH_2-terminal kinase (JNK).[12]

The evidence has shown that RNase L plays a role in cancer biology. The ARG462GLN variant of RNase L, which has an attenuated enzymatic activity, is implicated in up to 13% of prostate cancer cases. Individuals heterozygous for these mutations exhibit a 150% increased risk of prostate cancer, and homozygotes have greater than double of the risk, underscoring the importance of inactivating RNase L in the etiology of prostate cancer.[13–16] The inhibitory effect of RNase L on tumour formation is believed to be due in part to its anti-proliferative and pro-apoptotic roles. However, no spontaneous tumour formation has been observed in one-year-old RNase $L^{-/-}$ mice. The effect of RNase L on tumourigenesis induced by carcinogens is under investigation in our laboratory. Previously we have reported that the overexpression of RNase L in murine NIH 3T3 cells increased IFN anti-proliferative function.[5] In this study, we have found that bone marrow cells deficient in RNase L grew significantly faster compared to wild type cells in response to granulate macrophage colony stimulating factor (GM-CSF), suggesting that RNase L regulates cell proliferation stimulated by other growth factors. To determine the direct impact of RNase L on tumour growth in the absence of IFN-induced proteins, we have stably expressed RNase L in P-57 cells, an aggressive mouse fibrosarcoma cell line. To assess the effect of RNase L expression on the ability of p-57 cells to form tumours, these cells were implanted in athymic mice. Results showed that tumour formation was significantly delayed and the tumours grew much slower in the group with RNase L (P-RL) compared to the control group (P-Vec). Our findings suggest that RNase L plays a critical role in the inhibition of fibrosarcoma growth in nude mice.

2. Materials and methods

2.1. Cell culture

RNase $L^{+/+}$ and $^{-/-}$ mouse embryonic fibroblasts (MEF), P-57 cells (a gift from Dr. Chaoqun Wu, Fudan University), were grown in DMEM (The Media Lab of the Central Cell Service, Cleveland Clinic Foundation) supplemented with 10% foetal bovine serum (Biosource) and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C. Mouse bone marrow cells isolated from RNase $L^{+/+}$ and $^{-/-}$ mice were grown in RPMI-1640 supplemented with 10% foetal bovine serum and 10 ng/ml murine GM-CSF.

2.2. Overexpression of human RNase L in P-57 Cells

Murine fibrosarcoma cells (P-57) were transfected using Lipofactamine reagent (Invitrogen), with a mammalian expression vector, pcDNAneo (Invitrogen), inserted with or without human RNase L cDNA. The cells were selected in the medium containing G418 at 300 μg/ml. Twenty drug-resistant clones transfected with the human RNase L cDNA were isolated and analysed for RNase L expression by Western blot analysis. The clonal cell lines containing the vector with RNase L insert or an empty vector are referred to as P-RL and P-Vec, respectively.

2.3. Western blot analysis

Cells were harvested by washing twice with ice-cold phosphate-buffered saline (PBS) and collected with a scraper. Cytoplasmic extracts were prepared by the suspension of cell pellets in NP-40 lysis buffer (10 mM Tris–HCl, pH 8.0, 5 mM $Mg(OAc)_2$, 90 mM KCl, 0.2 mM PMSF, 100 U/ml aprotinin, 10 μg/ml leupeptin and 2% NP-40). After centrifugation at 10,000*g* in a microcentrifuge at 4 °C for 10 min, cell extracts (100 μg per sample) were fractionated on SDS–10% polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk in PBS containing 0.02% sodium azide and 0.2% (v/v) Tween 20, and incubated with a monoclonal antibody against human RNase L for 1 h at room temperature. The membranes were then washed with PBS containing 0.2% (v/v) Tween 20 and incubated with goat antimouse antibody conjugated with horseradish peroxidase (Cell Signaling) for 1 h at room temperature. After washing, RNase L was detected by a chemiluminescence method according to the manufacturer's specification (Amersham).

2.4. Assay for cell proliferation

RNase $L^{+/+}$ and $^{-/-}$ MEFs, P-Vec and P-RL cells (3×10^4) were seeded in 6-well plates, and after 1 d, they were treated with or without 1000 U/ml of murine IFN-α (Biosource). Fresh IFN was added again every other day. Viable cells were counted by trypan blue dye exclusion assays daily. The proliferation of RNase $L^{+/+}$ and $^{-/-}$ bone marrow cells was determined using the colorimetric CellTiter 96 aqueous Cell Proliferation Assay according to the instruction provided by the manufacturer (Promega). Briefly, cells (1×10^4 cells per well) were grown in 96-wells plates in the presence of 10 ng murine GM-CSF. At various times of cell growth, 50 μl CellTiter 96 Aqueous reagent (40% v/v dilution in 1 × PBS) was added to each well. Plates were incubated at 37 °C for 2 h, and absorbance was measured at 490 nm with a 96-well plate reader (Spectra Max 340; Molecular Devices).

2.5. Anchorage-independent growth assays

P-RL and P-Vec cells (1×10^4/ml) in 2× DMEM culture medium were mixed with an equal volume of 0.7% agar and plated onto 6-well plates with a layer of 0.6% bottom agar. The plates were incubated at 37 °C and 5%CO_2 in a humidified incubator for 10 days. The colonies were visualised under Olympus CK×31 at 100× magnification.

2.6. Tumour growth in vivo

P-RL and P-Vec cells (1×10^6) were injected subcutaneously into the flanks of groups of five NCRNU-M nude mice (Taconic Labs). Tumour volumes were measured weekly using a caliper ($H \times W \times D$) until the animals were euthanised.

2.7. Preparation of tumour tissue for microscopic examination

Tumours were excised and fixed in 10% Formalin (Fisher). The fixed tumours were then subjected to routine tissue processing. The paraffin embedded tumour tissues were thin sectioned at 5 μm and mounted on glass slides. Tissues were stained with hematoxylin and eosin.

3. Results

3.1. RNase L inhibition of cell proliferation

To determine the role of RNase L in the regulation of cell proliferation, growth rates of RNase L $^{+/+}$ and $^{-/-}$ MEF cells were compared in the presence or absence of 1000 U of IFN-α by

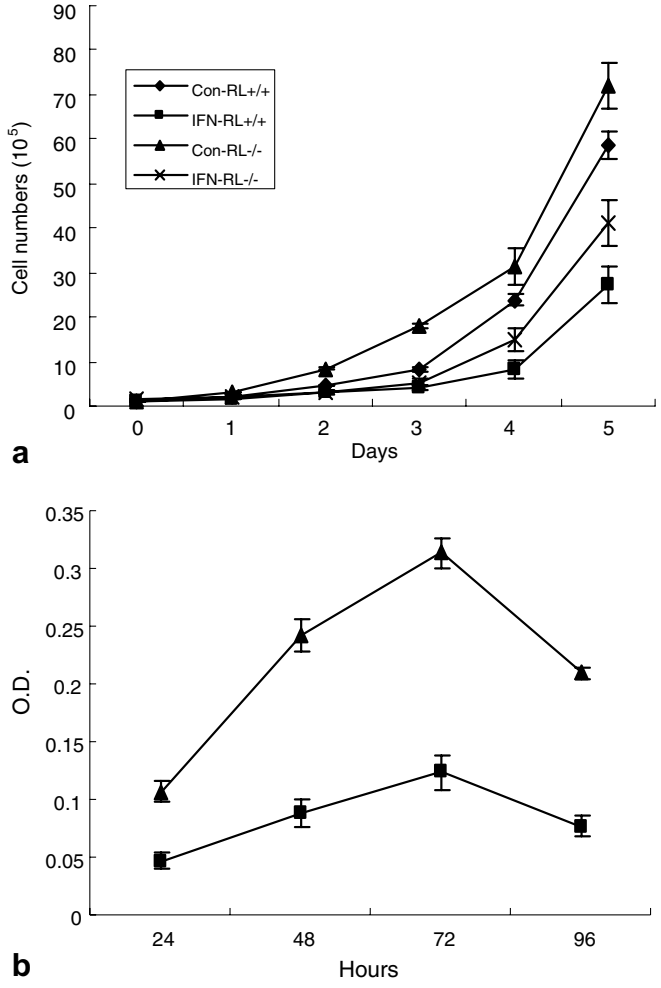

Fig. 1 – (a) Effect of RNase L on MEF proliferation. RNase $L^{+/+}$ and $^{-/-}$ MEF cells (1×10^4) were grown in DMEM supplemented with 10% FBS in six-well plates, and after 1 d, they were treated with or without 1000 U/ml of murine IFN-α (Biosource). The cells were trypsinised and counted daily. Experiments were performed in triplicate, and averages of the cell counts are presented, ±SD. Con-$RL^{+/+}$: untreated wild type MEF; IFN-$RL^{+/+}$: IFN-treated wild type MEF; Con-$RL^{-/-}$: untreated RNase L null MEF; IFN-$RL^{-/-}$: IFN-treated RNase L null MEF. (b) Effect of RNase L on murine bone marrow proliferation stimulated by GM-CSF. Bone marrow cells isolated from RNase $L^{+/+}$ and $^{-/-}$ mice were cultured in RPMI 1640 supplemented with 10% FBS and 10 ng/ml murine GM-CSF (Calbiochem). Cell proliferation was determined using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). A treatment group consisting of six wells and independent experiments were performed twice. Data represent means ± SD. (■) RNase $L^{+/+}$; (▲) RNase $L^{-/-}$.

measuring cell population numbers as a function of time. Both RNase $L^{+/+}$ and RNase $L^{-/-}$ MEF cells were susceptible to the anti-proliferative activity of IFN. There was a 2.5-fold reduction of growth rate in RNase $L^{+/+}$ MEF cells and a 2.0-fold reduction in RNase $L^{-/-}$ MEF cells in the presence of IFN (Fig. 1a). These results are consistent with our previous report that there are novel pathways of IFN action against viral infection and cell proliferation.[17] Surprisingly, without IFN treatment, RNase $L^{-/-}$ MEF cells grew 1.6-fold faster when compared to RNase $L^{+/+}$ cells. To demonstrate that the RNase L regulation of cell proliferation independent of IFN was not limited to specific cell type, the growth rates of bone marrow cells isolated from RNase $L^{+/+}$and $^{-/-}$ mice were compared in the presence of GM-CSF. As shown in Fig. 1b, RNase $L^{-/-}$ bone marrow cells grew 1.73-fold faster when compared to RNase $L^{+/+}$ bone marrow cells. This result supports a broader role for RNase L in the control of cellular proliferation.

3.2. *Expression of RNase L in the murine fibrosarcoma cell line P-57*

To directly measure the effect of RNase L on tumour cell growth in the absence of other IFN-induced proteins, we constitutively expressed huRNase L cDNA in the aggressive mouse fibroblastoma cell line P-57. The huRNase L cDNA was subcloned into the *Hind*III site of mammalian expression vector, pcDNAneo (Invitrogen). Transfection of the P-57 cells was followed by selection in media containing G418. Three of twenty G418-resistant clones (P-RL) were isolated that overexpressed RNase L by about 15–20-fold compared with the endogenous levels of murine RNase L in P-57 cells (Fig. 2a). The *in vitro* cell growth rates of the P-RL cells were 1.4-folds slower when compared to the clonal cell lines containing the empty vector (P-Vec) in the absence of IFN. However, the overexpression of RNase L seemed not to significantly contribute the anti-proliferative effect of IFN. IFN treatment reduced the growth rate at a similar ratio in both P-RL and P-Vec cells (2.6:2.2) (Fig. 2b). This result was apparently different from the previous observation in NIH 3T3 cells.[5] At low cell density, the morphologic difference between P-RL and P-Vec cells was not obvious. However, P-Vec cells continued to grow aggressively after the cells reached confluency. They piled onto each other and formed three-dimensional tumour-like masses with cells in elongated, spindle or fusiform shapes. On the contrary, P-RL cells displayed contact inhibition when they reached full confluence. The P-RL cells were large, round and polygonal (Fig. 3a). We performed a soft-agar colony-formation assay to determine the anchorage-independent growth of the two types of cells. As shown in Fig. 3b, colonies were clearly visualised in soft agar seeded with P-Vec cells after incubation for 10 d. In contrast, no colonies were formed in soft agar containing P-RL cells.

3.3. *RNase L inhibits tumour growth in vivo*

To determine the impact of RNase L on tumour growth *in vivo*, P-RL and P-Vec cells were injected subcutaneously into the flanks of groups of five nude mice (Taconic). Tumour growth was monitored weekly with a caliper. P-RL and P-Vec cells

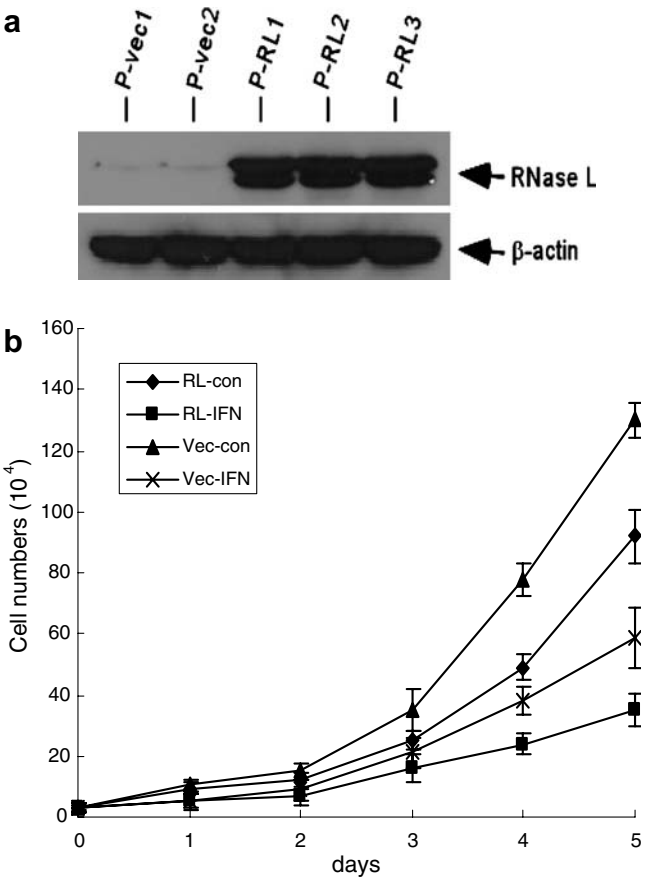

Fig. 2 – (a) Expression of human RNase L in murine P-57 fibrosarcoma cells. The huRNase L cDNA was subcloned into the *Hind*III site of mammalian expression vector, pcDNAneo (Invitrogen). Transfection of the P-57 cells was followed by selection in media containing G418. Expression of RNase L and β-actin in vector control clones (p-Vec1 and p-Vec2) and RNase L gene transfected clones (p-RL1, p-RL2 and p-RL) were analysed by Western blots using monoclonal antibodies to human RNase L and β-actin (Santa Cruz). (b) Effect of RNase L on P-57 cell proliferation. P-Vec and P-RL cells were grown in DMEM supplemented with 10% FBS in 6-well plates, and after 1 d, they were treated with or without 1000 U/ml of murine IFN-α (Biosource). The cells were trypsinised and counted daily. Experiments were performed in triplicate, and averages of the cell counts are presented; ±SD.

showed a dramatic difference in tumour growth (Fig. 4a). The formation of the P-RL tumours was 2 weeks delayed, and P-RL tumours grew markedly slower when compared with P-Vec tumours. However, after 5 weeks, the growth of P-RL tumours started to accelerate. Gross examination of tumours from the P-Vec cells showed that they were firm, fleshy, rounded or lobulated grey-white masses, with an average volume of 1295 mm^3 after five weeks. Although the tumours formed from the P-RL cells have similar gross appearance, they were much smaller. The average volume of the P-RL tumours was 99.2 mm^3 in dimension (Fig. 4b). To

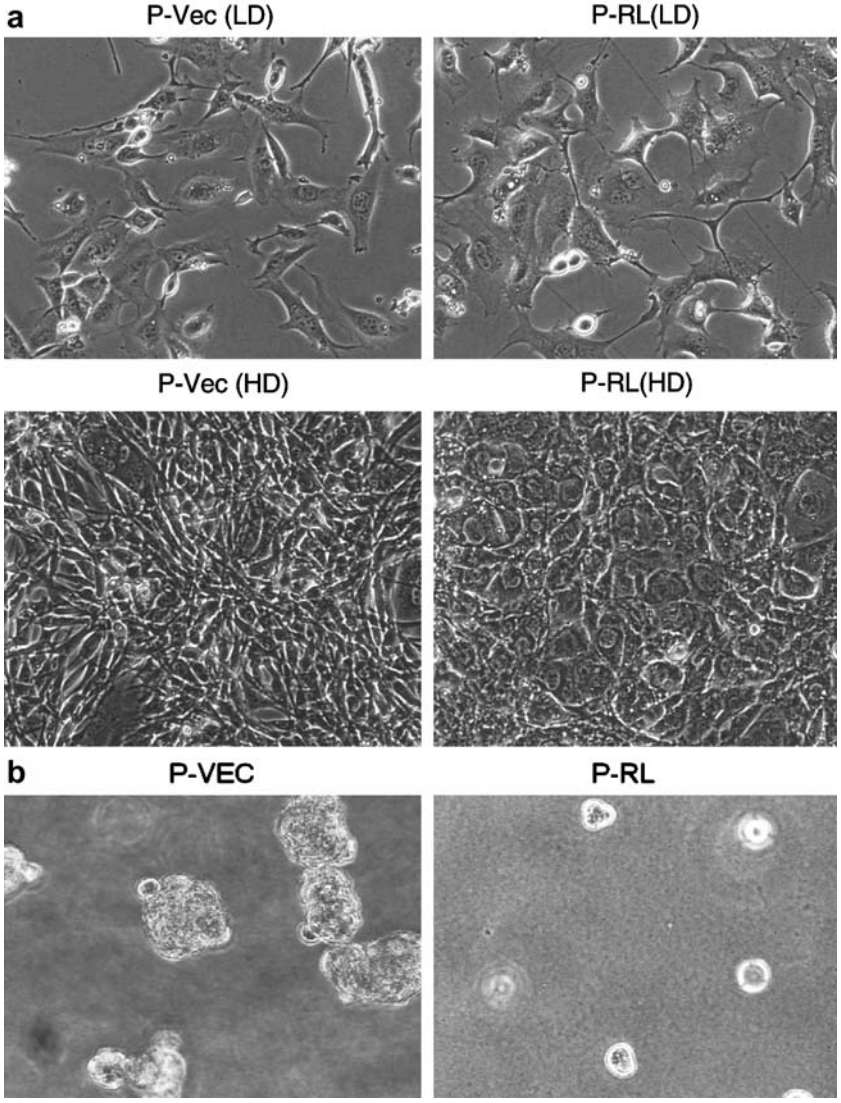

Fig. 3 – Morphology of P-RL and P-Vec cells. Photographs of the unstained cells at different growth stage (a) and colonies formed in soft agar were taken (b) under Olympus CK×31 at 100× magnification. LD: low density; HD: high density.

rule out clonal variation as the cause of the anti-tumour effect, we separately implanted groups of four mice with two other clones of P-RL cells. The tumour results obtained from the different clones of P-RL cells (data not shown) were similar to that from the first clone used for the experiment, thus eliminating clonal differences as the basis for the anti-tumour activity of RNase L.

Microscopically, the P-Vec tumours demonstrated a uniform herringbone growth pattern consisting of spindle-shaped cells that varied little in size and shape and had scant cytoplasm with indistinct cell borders (Fig. 5a). Interestingly, dramatic differences in morphology were observed in P-RL tumours (Fig. 5b), which had a less distinct fascicular or herringbone pattern. The tumour cells were epithelioid with large round and polygonal cells, open chromatin, and prominent central nucleoi. They also showed prominent pleomorphism with variable cell shape and nuclear size. This morphologic difference resembled that of the cultured cells (Fig. 3a).

3.4. *P-RL tumour growth is due to suppression of RNase L expression*

It was possible that P-RL tumours started to grow eventually in the nude mice were due to the suppression of RNase L expression by an unknown mechanism. To determine RNase L level in the tumour cells, RNase L in P-RL tumours was measured by Western blot analysis in tumour extracts using a monoclonal antibody to human RNase L. As shown, the expression of RNase L was completely shut-down in the P-RL tumours, suggesting that RNase L plays an important role in the inhibition of tumour growth (Fig. 6).

4. Discussion

RNase L has been suggested to function as a tumour suppressor based on its roles in mediating apoptosis and anti-proliferative activity of IFN.[18] Our findings provide the first direct evidence that RNase L is able to inhibit tumour growth

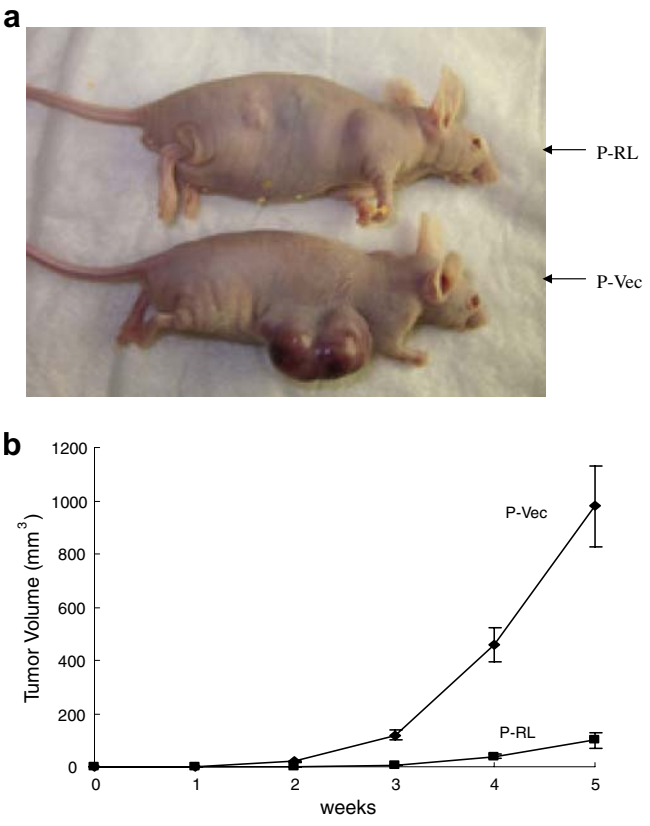

Fig. 4 – Overexpression of RNase L inhibits tumour growth. P-57 cells (10^6/site) transfected with pcDNAneo (P-Vec) or pcDNAneo/RNase L (P-RL) were injected subcutaneously into the flanks of groups of three nude mice. (a) Representative tumours and mice on day 30 after implantation with P-Vec and P-RL cells were shown. (b) Tumour volumes were determined using a caliper. Data are presented in means ± SD. $P < 0.003$ on day 30 in a paired Student's t-test.

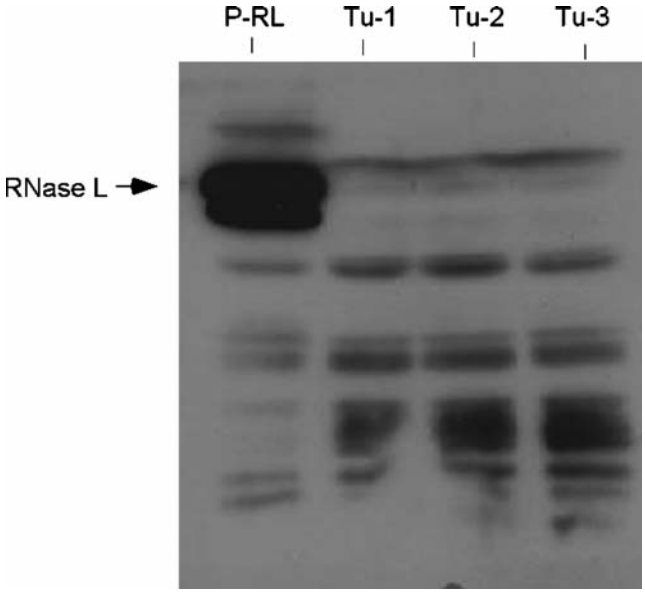

Fig. 6 – Suppression of RNase L expression in tumour. P-RL tumours on day 36 were dissected and homogenised in NP-40 buffer. The expression of RNase L was determined by Western blot with a monoclonal antibody against human RNase L. P-RL: P-RL cells; Tu 1–3: P-RL tumor 1–3.

in vivo. This result is consistent with the observation that mutations in the RNase L gene predispose men to an increased incidence of prostate cancer.[13–16] Interestingly, P-RL cells eventually start to grow into tumours as a result of completely blocking the expression of RNase L, further confirming the tumour suppressive function of RNase L.

It has been demonstrated that RNase L plays an important role in the effect of IFNs against viral infections and cellular proliferation. The activators of RNase L, 2-5A, are generated from ATP by the dsRNA-activated 2-5A synthetases, a family of enzymes induced by the IFN treatment of mammalian cells. The only well-established function of 2-5A is to activate RNase L, that subsequently cleaves single strand RNA, resulting in the inhibition of both viral replication and cellular proliferation. Natural 2-5A molecules are very unstable and degraded by a combination of 5′-phosphatase and 2′,5′-phosphodiesterase present in cells. Therefore, in the absence of IFN and viral infection, 2-5A levels are either very low or absent. Our results have shown that although IFN treatment

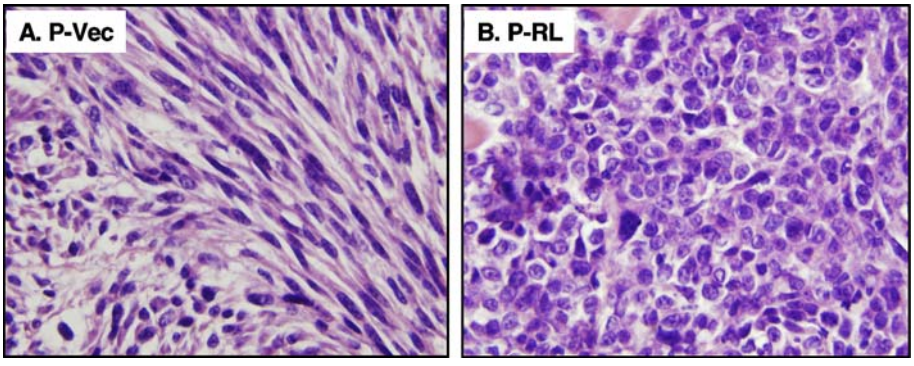

Fig. 5 – Distinctive cellular morphology of P-Vec and P-RL tumours. P-Vec and P-RL tumours recovered by necropsy at day 30 after cell implantation were fixed in 10% Formalin, routinely processed and sectioned, and stained with H&E. Photomicrographs (600×) shown here are representative of multiple fields examined in tumours excises from two independent experiments.

produces an enhanced anti-proliferative effect on MEFs, RNase L is able to inhibit cell proliferation independent of IFN (Fig. 1a). In addition, RNase L also regulates the growth of bone marrow cells in the presence of GM-CSF, suggesting a broader role of RNase L in mediating cell proliferation (Fig. 1b). Surprisingly, we found that the overexpression of RNase L suppressed the tumour cell growth although there was no significant difference between P-RL and P-Vec cells in response to IFN (Fig. 2b). The expression of RNase L is cell specific. The expression level of RNase L in certain cell types such as B-cells, lymphoblasts, myelobasts and monocytes is about 20–30-fold higher than that in fibroblasts (our unpublished data). How these cells survive with the extremely high level of RNase L remains to be investigated.

RNase L is believed to display its biological function through regulating mRNA stability after IFN treatment. Ribosomal and viral RNAs have been the first reported targets of RNase L. Documented evidence has shown that RNase L plays an important role in the stability of several gene products, including IFN-induced genes, such as ISG43, ISG15[19] and PKR[20] in RNase L null cells; MyoD mRNA in myocytes[21] and mitochondrial mRNAs in H9 lymphocytes.[22] However, in the absence of viral infection, the sources of dsRNA and 2-5A to activate 2-5A synthetase, and RNase-L remain obscure. It is possible that RNase L can indirectly regulate gene expression through interacting with other proteins because RNase L contains structurally nine ankyrin repeats, a typical protein–protein interaction domain. Recently, Peltz's group has demonstrated that RNase L is indeed able to regulate gene expression by interacting with another protein.[23] They discovered that RNase L can interact with human translation termination factor eRF3/GSPT1 to modulate the translation termination process, resulting in regulating gene expression. Their results outline a novel role for RNase L as a regulator of translation.

The effect of RNase L on cell growth was not obvious when cells were grown at a low density (Fig. 3a). However, when cells grew at a high density, the morphological difference between these two cell lines was dramatic. P-Vec cells grew more aggressively. Even though the cells have grown to confluence, P-Vec cells continued to grow, and piled up to form individual clones if fresh medium was provided. P-RL cells ceased growing after cells became confluent. These cells were large and had a polygonal shape, probably due in part to the altered cell morphology. Furthermore, anchorage-independent growth in soft agar (Fig. 3b) is consistent with the observation in tumour formation in nude mice (Fig. 4a). These findings indicate a role of RNase L in suppressing tumour growth. Surprisingly, no spontaneous tumour formation has been observed in one-year-old RNase $L^{-/-}$ mice. Currently, the effect of RNase on tumourigenesis induced by carcinogens is under investigation in our laboratory. We are also cross-breeding RNase $L^{-/-}$ with P53 $^{-/-}$ mice to determine whether $P53^{-/-}$ mice deficient RNase L results in an increase of spontaneous tumour formation.

The P-Vec tumours showed typical fibrosarcoma histology with fascicular, herringbone growth pattern and slender spindled cells. However, with the overexpression of RNase L, the tumour cells demonstrated polymorphism with more epithelioid cells. Since RNase L is involved in the regulating the activation of the JNK pathway, which roles in the control of cell motility and morphogenesis have been well established,[24] overexpression of RNase L may affect JNK-regulated cytoskeletal molecules, and therefore cause the morphological alteration. Interestingly, increased single cell necrosis was observed in P-RL tumours. Whether the increased cell death is due to the overexpression of RNase L promoting apoptosis needs to be further investigated.[9–11]

Conflict of interest statement

None declared.

Acknowledgements

We are grateful to Dr. Crystal Weyman for the helpful comments on the manuscript. This work was supported by the American Cancer Society Cleveland Pilot Grant and Established Full-time Faculty Research Development Award from Cleveland State University to A.Z.

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