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Insulin-like growth factor I receptor is expressed at normal levels in Nijmegen breakage syndrome cells

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Abstract

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder sharing a pleiotropic phenotype with ataxia-telangiectasia (A-T), including increased radiosensitivity and cancer disposition. Insulin-like growth factor I receptor (IGF-IR) expression is reportedly decreased in A-T cells, which is thought to contribute to its increased radiosensitivity. In this study, we investigated whether the same mechanism underlies the radiosensitivity of NBS cells. GM7166VA7 cells lacking NBS1 protein displayed a phenotype of increased radiosensitivity, while the introduction of NBS1 cDNA conferred radioresistance comparable to normal cells. IGF-IR expression levels were essentially the same among normal, NBS, and NBS1-complemented NBS cells. There was no significant difference between NBS and NBS1-complemented cells in activation of major downstream pathways of IGF-IR upon IGF-I stimulation, including phosphatidylinositol-3′ kinase (PI3-K) and mitogen-activated protein kinase (MAPK). Collectively, IGF-IR-related events are unlikely to be disrupted in NBS cells, and therefore, defects in IGF-IR signaling do not explain the increased radiosensitivity of NBS cells.

Keywords: Nijmegen breakage syndrome; Ataxia-telangiectasia; Insulin-like growth factor I receptor; Radiosensitivity

Ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) are autosomal recessive disorders with strong parallels in the pattern of radiosensitivity and cancer disposition [1,2]. The gene product mutated in A-T patients, designated ATM, was found to be a member of the phosphatidyinositol-3′ kinase-related kinase family [3] and functions as an upstream sensor of DNA damage, especially following ionizing radiation [1]. ATM also regulates cell cycle checkpoints through p53 and Chk2 [4]. Recently, ATM was demonstrated to phosphorylate NBS1, the gene product mutated in NBS, in response to DNA damage by ionizing radiation [5,6]. NBS1 co-associates with hMre11 and hRad50 and is a functional homolog of xrs-2, albeit sharing only limited amino acid sequence homology [7]. The ATM/NBS1 pathway is thus likely to contribute to proper double strand break (DSB) repair through homologous recombination or non-homologous end-joining [1].

The insulin-like growth factor I receptor (IGF-IR) plays a pivotal role in cell growth, transformation, differentiation, and protection from apoptosis [8–10]. A variety of stimuli induce apoptosis and IGF-IR is reported to confer a strong anti-apoptotic activity [8,11]. Nevertheless, thus far only limited reports are available

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concerning the effects of IGF-IR on ionizing radiation-induced cell death [12–14]. Peretz et al. [15] reported that IGF-IR expression levels are suppressed in A-T cells and that the transcriptional activity of the IGF-IR gene is dependent on ATM activity. Furthermore, overexpression of IGF-IR in A-T cells restored its radiosensitivity. Therefore, it was concluded that reduced IGF-IR levels contribute significantly to the extreme radiosensitivity of A-T cells [15]. Here, we examine the expression and major downstream signaling of IGF-IR in NBS patient fibroblasts.

Materials and methods

Cell lines and culture conditions. All the cell lines used in the present study were SV 40-transformed human fibroblasts. MRC5VA (supplied from Riken Cell Bank) and LM217 [16] cells were derived from normal individuals. GM7166VA7 cells were derived from an NBS patient [17] and GM7166VA + NBSmix cells were established from GM7166VA7 cells by stable transfection of a plasmid carrying wild type NBS1 cDNA, as described previously [17]. Some clones were also obtained from mixed populations. P6 cells overexpressing human IGF-IR [18] were used as a positive control in some experiments. All cell lines were maintained in Eagle's minimum essential medium (MEM) containing 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin supplemented with 10% fatal bovine serum in a humidified atmosphere containing 5% CO_2 and 95% air.

^{125}I-IGF-I binding assay. IGF-I binding sites were determined in each cell line by an ^{125}I-IGF-I binding assay, as described previously [19]. Briefly, cells were incubated in a binding buffer containing 0.5 ng/ml of ^{125}I-IGF-I (Amersham, Arlington Heights, IL) for 4 h at 4 °C. After washing with cold HBSS, cell-associated radioactivity was measured with an autowell γ-counter. Specific binding was expressed by subtracting nonspecific binding (determined in the presence of 400-fold excess of unlabeled IGF-I) from total binding.

Western blotting. Western blotting was performed as described previously [13]. Primary antibodies used for detection of IGF-IR β-subunit, NBS1, and β-actin were an anti-IGF-IR Ab (c-20), an anti-Nibrin Ab (c-19) (Santa Cruz Biotech, Santa Cruz, CA), and an anti-β-actin Ab (Chemicon International, Temecula, CA), respectively. For detection of the active form of ERK, an anti-active MAPK Ab (Promega, Madison, WI) was used.

Clonogenic cell survival assay. Exponentially growing cells were trypsinized and re-suspended cells were sealed in a plastic tube. Cells were irradiated using a ^{60}Co γ-ray therapeutic machine, RCR-120 (Toshiba, Tokyo, Japan) at a dose rate of 1.47 Gy/min. After irradiation, an appropriate number of cells were plated in tissue culture dishes. After incubation for 12–14 days, cells were fixed and stained with crystal-violet. Colonies containing more than 50 cells were counted and surviving fractions were determined.

Kinase assay for phosphatidylinositol-3′ kinase. PI3-K assay was performed as described previously using phosphatidylinositol (Avanti, Alabaster, AL) and [γ-^{32}P]ATP (Amersham) as substrates [20]. Autoradiograms were obtained after separation of phosphatidylinositol phosphates (PIP) by thin-layer chromatography. Quantitation of PIP was performed by a scintillation counter.

Kinase assay for MAPK. Kinase assay for MAPK was performed according to the method of Kaburagi et al. [21]. Briefly, serum- starved cells were stimulated with IGF-I and lysed. MAPK activity in the cell lysates was determined using myelin basic protein (Sigma Aldrich, St. Louis, MO) and [γ-^{32}P]ATP as substrates.

Statistics. The unpaired Student's *t* test was used to analyze differences in mean values obtained from three independent experiments.

Results and discussion

GM7166VA7 cells were derived from a patient harboring a homozygous mutation, 657del5, in the NBS1 gene, resulting in a frameshift at codon 219 [22,23]. Expression of NBS1 protein in this cell line is reportedly undetectable [17], which was confirmed in this study by Western blotting (Fig. 1Aa). Mixed populations of GM7166VA7 cells transfected with the pIRES-NBS1 plasmid expressed NBS1 protein at similar levels to two different normal cell lines (Fig. 1Aa). Two clones derived from mixed populations also expressed similar levels to NBS1 protein (Fig. 1Ab). Dose-survival curves determined by colony-forming assay (Fig. 1B) confirmed that GM7166VA7 is highly radiosensitive and that NBS1-complementation, in mixed populations and clones, almost completely restored radioresistance, as shown in a previous report [17].

Using SV40-transformed human fibroblast cell lines, Peretz et al. [15] reported significantly reduced IGF-IR expression levels in A-T cells compared to normal cells, due to deficient ATM-dependent transcriptional activity of the IGF-IR gene. Since NBS is considered an A-T variant [1,2], we measured IGF-IR expression levels in NBS cells by ^{125}I-IGF-I binding assay (Fig. 2A) and Western blotting (Fig. 2B) in comparison with those of normal and NBS1-complemented cell lines. There was

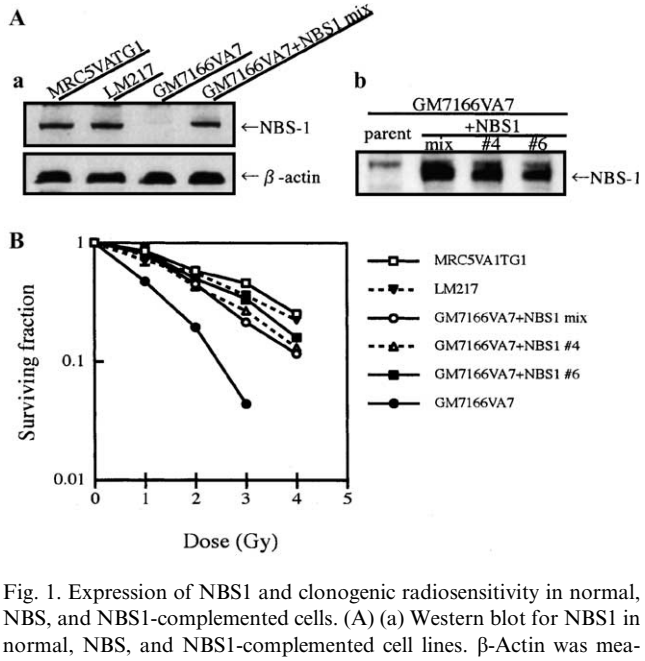

Fig. 1. Expression of NBS1 and clonogenic radiosensitivity in normal, NBS, and NBS1-complemented cells. (A) (a) Western blot for NBS1 in normal, NBS, and NBS1-complemented cell lines. β-Actin was measured as a loading control. (b) Western blot for NBS1 in NBS1-complemented cell lines (mixed populations and clones). (B) Dose-survival curves in normal, NBS, and NBS1 complemented cells. Exponentially growing cells were γ-irradiated and surviving fractions were determined as described in Materials and methods. The data represent the means ± SD of triplicate plates from a representative experiment. Bars are shown only when larger than symbols.

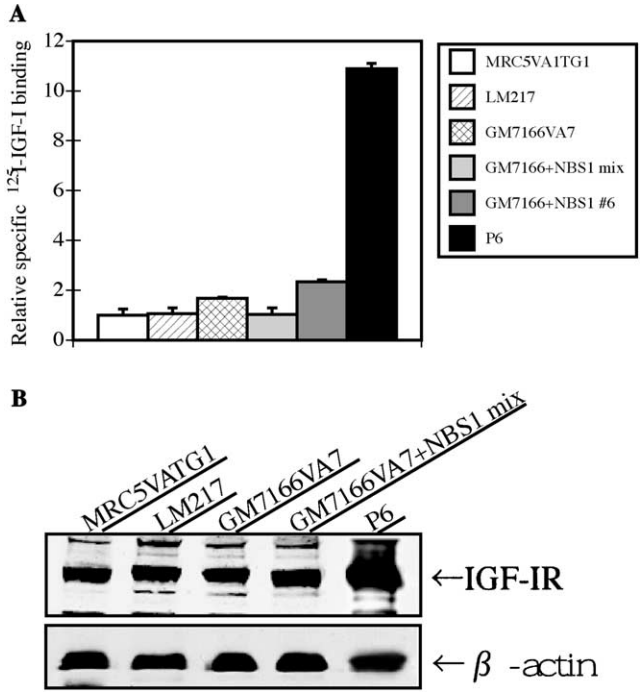

Fig. 2. Expression of IGF-IR in normal, NBS, and NBS1-complemented cells. (A) ^{125}I-IGF-I binding assay. Cells grown on six well-plates were incubated with 0.5 ng/ml of ^{125}I-IGF-I and specific cell surface binding was determined as described in Materials and methods. The data represent the means ± SD of three independent experiments. P6 cells were used as a positive control. (B) Western blot of IGF-IR in normal, NBS, and NBS1-complemented cell lines.

no evidence that IGF-IR levels were significantly decreased in NBS cells, although some variations were detected among isogenic NBS cell lines. These observations clearly contrast the characteristics of A-T cells reported by Perez et al. [15]. Semi-quantitative RT-PCR analysis of IGF-IR gene expression also indicated similar expression among normal, NBS, and NBS-complemented cell lines (data not shown).

We next assessed the downstream signaling activation of IGF-IR upon IGF-I stimulation. Signaling through IGF-IR is known to stimulate the MAPK pathway, and its continuous activation is implicated in cell growth and protection from apoptosis [24,25]. Phosphorylation of ERK-1, 2 was detected in all cell lines examined 10 and 60 min after IGF-I stimulation (Fig. 3Aa), indicating that IGF-IR mediated stimulation of MAPK is normal in NBS cells. A kinase assay using myelin basic protein as a substrate also showed similar activity between normal and NBS cells (Fig. 3Ab). Activation of another major downstream pathway, PI3-K, was also examined (Fig. 3Ba). The NBS cells showed rather less PI3-K activation upon IGF-I stimulation than normal cells, in a PI3-K assay using phosphatidylinositol as a substrate (Fig. 3Bb). However, isogenic NBS cells expressing exogenously introduced NBS1 exhibited similar PI3-K activity to NBS cells, demonstrating that a deficiency in NBS1 does not affect IGF-IR-mediated PI3-K activity (Fig. 3Bb).

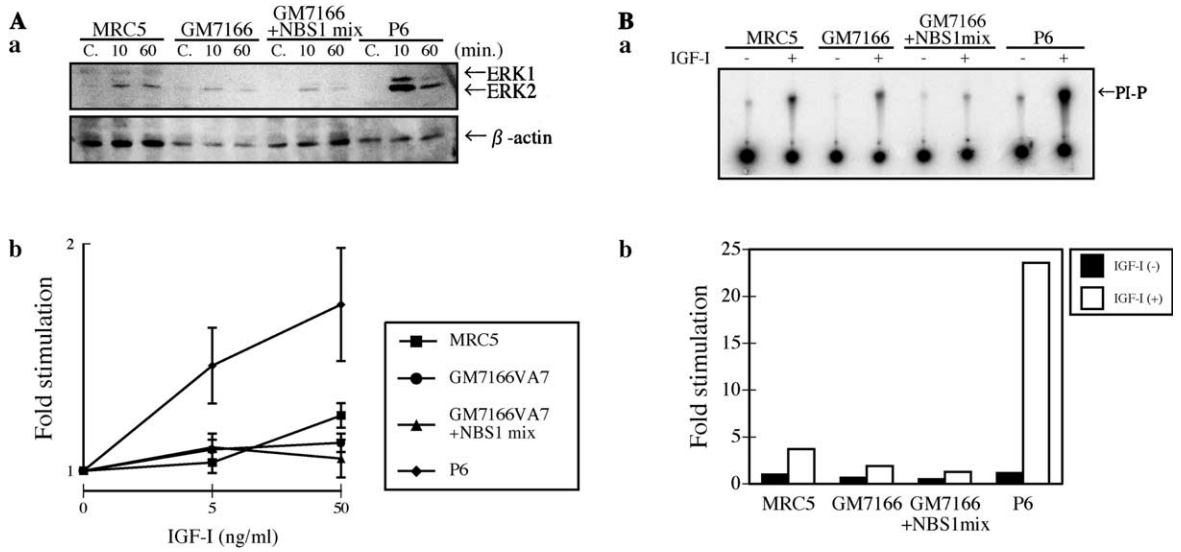

Fig. 3. Downstream signaling of IGF-IR in normal, NBS, and NBS1-complemented cells. (A) Activation of the MAPK pathway upon IGF-I stimulation. (a) Phosphorylation of ERKs upon IGF-I stimulation. Serum-starved cells were stimulated with IGF-I (50 ng/ ml) for 10 or 60 min and cell lysates were subjected to SDS–PAGE. Phosphorylated ERKs were detected as described in Materials and methods. C represents unstimulated controls. P6 cells were used as a positive control. (b) Kinase assay for MAPK. MAPK activity was also determined using myelin basic protein and ATP as substrates. Serum-starved cells were stimulated with 5 or 50 ng/ml of IGF-I for 10 min and cell lysates were analyzed as described in Materials and methods. The data were expressed as fold stimulation with each unstimulated value normalized to 1. The bars represent the means ± SD of three independent experiments. (B) Activation of PI3-K pathway upon IGF-I stimulation. (a) Autoradiogram of phosphatidylinositol phosphates (PIP). (b) Quantitation of PIP with the value of unstimulated MRC5VA cells normalized to 1.

The rationale behind the present study was based on the observation that A-T cells express decreased levels of IGF-IR and that forced expression of IGF-IR greatly improved the radiosensitivity [15]. Since NBS shares some clinical and biological features with A-T [1,2], we next investigated if the same mechanism also underlies the radiosensitivity of NBS cells. Unlike A-T cells as described by Perez et al. [15], NBS cells showed similar IGF-IR expression to normal cells (Fig. 2). Considering that expression of exogenous NBS1 almost completely normalized the radiosensitivity of NBS cells, it appears that NBS1 can function normally in GM7166VA7 cells. One may argue that IGF-IR expression is modified by SV40 transformation. The likelihood, however, is remote as expression of SV 40 T-antigen in these cell lines was variable (data not shown), and, furthermore, SV40 has been shown to enhance IGF-I, but not IGF-IR gene expression [26]. Indeed, primary MRC5 and SV40 transformed MRC5VA cells expressed the same levels of IGF-IR (data not shown).

Although A-T and NBS share some phenotypic characteristics, as described above, they also have significant differences [1,2,27]. The discrepancy in IGF-IR expression in patients with these two diseases may account for the phenotypic differences. While both types of patients exhibit high cancer predisposition, the actual incidence is much higher in NBS [27]. IGF-IR is known to play an important role in transformation or tumorigenesis [9], which may be relevant to the higher frequency of carcinogenesis in NBS in comparison with A-T patients. In addition, growth retardation in childhood is observed in NBS, but not in A-T [27,28], which is paradoxical, considering that IGF-IR stimulates growth [29]. Because IGF-IR has been shown to serve as a survival and differentiation factor for neurons [10], the phenotype of cerebellar ataxia observed only in A-T patients [1,2,28] may be explained by the decreased levels of IGF-IR, although cerebellar morphology was unaltered in mice with a disruption of the IGF-I gene [10].

It is still not known how ATM activates the IGF-IR promoter [15], however, NBS1 is unlikely to be required in this pathway. Although ATM and NBS1 share a common pathway in response to DNA damage, leading to cell cycle checkpoint control and double strand break repair [1], regulation of IGF-IR expression may be a unique function of ATM, for which NBS1 is irrelevant. Therefore, it is likely that the increased radiosensitivity found in cells with a disruption of NBS1 may be independent of IGF-IR-related events.

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