Reoviral Oncotropism Against c-Myc Overexpressing HS 68 Cells

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Naturally occurring reoviruses are live replication-proficient viruses specifically infecting human cancer cell while sparing normal counterpart. Since the discovery of reoviruses in 1950s, reoviruses have shown various degrees of safety and efficacy in pre-clinical or clinical application for human anti-cancer therapeutics. I have recently shown that cellular tumor suppressor genes, such as p53, ATM (Ataxia telangiectasia mutated), and RB (Retinoblastoma associated), are important in determining reoviral oncotropism. Thus, it is interesting to examine whether the aberrancy of c-Myc expression, whose normal function also plays an important role in the maintenance of genomic integrity, could affect reoviral oncolytic tropism. Hs68 cells are non-tumorigenic normal cells and resistant to reoviral cytopathic effects. Importantly, I found that c-Myc overexpression in human HS68 cells effectively induced reovirus cytopathic effects compared to mock expressed cells as shown by the typical reoviral cytopathology and an increased level of caspase-3 activity. Taken together, overexpression of c-Myc could play an important role in determining reoviral oncolytic tropism.

Key Words: Oncolytic virus; Reovirus; c-myc; Oncogenes; Reovirus oncolysis

INTRODUCTION

Reoviruses, which belong to reoviridae family, are ubiquitous, non-enveloped viruses containing 10 segments of double-stranded RNA (dsRNA) as their genome. Reoviruses are ubiquitous viruses that have been isolated from various sources such as a wide variety of mammalian species including humans as well as water and sewage (1). There are three serotypes of reoviruses based on their hemagglutination activity. Prototypical laboratory strains of each serotype were isolated from children's respiratory and enteric tracts and are designated Type 1 Lang, Type 2 Jones, and Type 3 Dearing (2). Reovirus type 3 Dearing stain is currently used as a natural oncolytic virus for various preclinical studies as well as human clinical trials (3).

Oncolytic viruses are live replication-proficient viruses specifically infecting human cancer cell while sparing normal counterpart. Oncolytic viruses are quite different from conventional gene therapy viral vectors in several aspects. Firstly, oncolytic viruses do not compromise their replication potential while gene therapy vectors almost always modify viral genes to compromise viral replication potential. Secondly, oncolytic viruses sometimes undergo viral attenuation to reduce their natural toxicity while gene therapy vectors do not need viral attenuation since viral vectors are already replication incompetent. Lastly, oncolytic viruses can contain a foreign gene, which can be expressed, in their viral genome without compromising viral replication potential while gene therapy vectors usually contain a foreign
gene, which should be expressed, in their viral genome with lack of replication potential (4).

Because of these unique features of the replicating nature of oncolytic viruses, they are highly dependent on host cell physiology for optimal performance as viral cancer-targeting agents. Many naturally occurring viruses have shown a great potential as cancer targeting agents by exploiting various oncogene signalling pathways established by host cancer cells during tumorigenesis (5–7).

c-Myc proto-oncogene plays an important role in genomic maintenance and deregulated c-Myc expression generates genomic instability (8–10). Oncogenes such as Ras and c-Myc were initially to be oncogenic solely because of their role in proliferative control. However, recent studies proposed that chromosome instability caused by combined dysfunctional effects of oncogenes and tumor suppressor genes may be more central to tumorigenesis than previously thought (11). Thus, not only oncogenes but also tumor suppressor genes may play an important role in determining the oncolytic nature of a replicating virus since genomic instability could compromise integrity of normal cellular anti-viral networks. Because carcinogenesis is a multi-step process involving accumulation of not only oncogene abnormality but also accumulation of tumor suppressor gene abnormality, I recently discovered that cellular tumor suppressor genes are also important in determining oncolytic viral tropism including reoviruses (12). Thus, mechanistically reovirus oncolysis can be established by both cellular oncogene/tumor suppressor gene abnormalities.

Since the discovery of reoviruses in 1950s, reoviruses have shown various degrees of safety and efficacy in preclinical or clinical application for human anti-cancer therapeutics. In order to further elaborate mechanistic basis of inherent reovirus oncotropism, I examined whether aberrant c-Myc expression could affect reovirus oncolytic tropism.

**MATERIALS AND METHODS**

**Cells and virus stocks**

HS68 cells were purchased from ATCC (Manassas, VA, USA). These were also routinely grown in High-Glucose DMEM, supplemented with 10% fetal bovine serum. For experiments involving high c-Myc expression, we used Adenoviral expression system provided by Wafik S. El-Deiry's lab in Fox Chase Cancer Center. c-Myc expressing adenoviral vectors are propagated on 293 monolayers according to the protocol provided by Adenoviral expression system (Santa Clara, CA, USA). Hs68 cells were infected either with a non-replicating adenovirus vector expressing human c-Myc driven from a CMV promotor and GFP driven from a separate promotor (Ad-Myc), or with the same virus construct expressing GFP only (Ad), as a negative control. HS68 cells were infected with the Ad-Myc or Ad viruses 6 to 12 hours prior to reovirus infection. Reovirus serotype 3 Dearing Strain was grown on L929 cells as described previously (13). Plaque titrations were carried out in duplicate on L929 monolayers as described previously (13).

**Infection of cells and Western Blotting**

Subconfluent plates of cells were infected with reovirus type 3 Dearing at 20 multiplicities of infection (MOI) in 1 ml/10 cm plate of complete medium, and virus adsorption was carried out for 45 min. at room temperature. Following adsorption, 8ml of fresh medium was added to each plate. To harvest for western blot analysis, cells were scraped from the plates, washed extensively with PBS and lysed in RIPA Buffer (50 Mm Tris pH 7.2, 150 Mm NaCl, 0.5% Sodium Deoxycholate, 0.1% SDS, 1.0% Triton-X 100, supplemented with protease and phosphatase inhibitors immediately prior to use). Lysates were clarified by centrifugation and protein concentrations in the lysates were determined by Bradford Assay (Bio-Rad). Aliquots of whole cell lysate were separated on 12% SDS-PAGE gels, transferred to nitrocellulose, probed with the appropriate antibodies and developed using enhanced chemiluminescence (Pharmacia).

**Antibodies**

The anti-c-Myc monoclonal antibody specific for human c-Myc was the 9E10 clone from BioLegend (Dedham, USA). Anti-polyclonal reovirus antiserum was provided by
Patrick Lee's lab in Dalhousie University.

**Determination of caspase 3 activity**

Cells were infected as described above and harvested and the lysates assayed for caspase 3 activity using a caspase 3 Colorimetric Assay Kit from Sigma Aldrich. This caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm. Caspase 3 activity was determined by measuring the absorbance at 405 nm of whole cell lysates at various times following addition of the peptide substrate. Lysates were assayed in triplicate.

**RESULTS**

Reovirus effectively induces cytopathology in c-Myc expressing HS68 cells

To investigate the reoviral effect on c-Myc expressing human cells, normal human fibroblasts (HS68 cells) were infected with either an Ad-control vector or an Ad-Myc vector. Cells were infected with these vectors and allowed to accumulate high levels of c-Myc for 24 hours before they were challenged with reovirus type 3 Dearing strain. Cells were harvested 48 hours post-infection and the lysates examined for the levels of c-Myc protein expression. Fig. 1A shows the expected high levels of c-Myc expression only in cells exposed to the Ad-Myc vectors. When c-Myc expressing Hs68 cells were challenged with reovirus, cells showed a typical reoviral cytopathic effects (cell rounding and floating) as shown in Fig. 1B. Importantly, c-Myc
expression alone does not induce viral cytopathic effects. Taken together, virally resistant Hs68 cells become susceptible to reovirus when cells are ectopically expressed with c-Myc.

**c-Myc overexpression induces an increased level of caspase-3 activity upon reovirus exposure**

As c-Myc has been shown to be capable of apoptotic induction, caspase-3 activity level was investigated upon Ad-Myc transduction since this death executioner caspase activity is thought to reflect relatively a late stage of apoptosis (14). Since virally resistant Hs68 cells become susceptible to reovirus upon c-Myc overexpression, I examined whether c-Myc overexpression could affect reoviral production level. Thus, Hs68 cells were exposed to increasing amounts of the Ad-Myc vector from 0 MOI to 100 MOI prior to being infected with reovirus at an MOI of 20. Cells were then harvested at 48 hours post-infection and probed for reoviral structural proteins of lambda, mu and sigma. Interestingly, the amounts of viral protein in the infected cells remained about the same at 48 hours post-infection regardless of the expression levels of c-Myc (Fig. 2A). However, caspase 3 activity is significantly increased in the higher level of c-Myc expressing cells as shown in Fig. 1B. Taken together, c-Myc overexpression renders cell to become susceptible to reovirus infection via the death caspase activation although reoviral infection efficiency may not be affected by c-Myc overexpression.

**DISCUSSION**

Since the discovery of reoviruses in 1950s, oncolytic nature of several reovirus strains have been identified. During the past 15 years of molecular works, involvement of cellular oncogenes and tumor suppressor genes in determining reoviral oncolytic tropism is now fairly well established (7, 12). In this study, we propose that c-Myc oncogene also play an important role in determine inherent reovirus oncotropism. I have recently reported that cellular tumor suppressor genes, such as p53, ATM, and RB, are important in determining reoviral oncotropism (12). These tumor suppressor genes are known to play important roles in genomic fidelity/maintenance. Thus tumor suppressor gene abnormality could affect host genomic integrity and likely disrupt intact antiviral networks due to the accumulation of genetic defects, which would in turn results in oncolytic reovirus susceptibility. Thus, it is interesting to examine whether aberrant expression of c-Myc, whose normal function also plays an important role in the maintenance of...
genomic integrity (8–10), could affect reoviral oncolytic tropism. c-Myc activation is a hallmark of cancer initiation and maintenance (15).

Using adenoviral c-Myc expression system, I found that c-Myc overexpression in human HS68 cells effectively induced reovirus oncolytic effects compared to mock expressed cells as shown by the viral cytopathology and an increased level of caspase-3 activity, indicating that c-Myc plays an important role in determining reovirus oncolytic tropism. Because the hyper-activation of c-Myc is frequently found in a variety of human cancers (16), reovirus could be an effective oncolytic agent targeting c-Myc overexpressing human cancers.

c-Myc overexpressions are frequently found in a variety of human cancers. For instance, ovarian cancer is known to have frequent genetic abnormalities resulting in over-expression of c-Myc and c-Myc overexpressing ovarian cancers have a poor prognosis (17, 18). In 2002, Hirasawa et al reported that reovirus type 3 Dearing strain is highly effective in suppressing ovarian tumors in a xenografted animal model (19). Recently, reovirus type 3 Dearing strain was administrated to ovarian tumor patients in clinical trials (20, 21). Thus, safety and efficacy of reovirus therapy against intractable ovarian cancer could be reported in the near future.

REFERENCES

