Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 582-587

A SIMPLE AND EFFICIENT METHOD OF PROTEIN DELIVERY INTO CELLS USING ADENOVIRUS

Prem Seth[1]

Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis, National Cancer Institute, Bethesda, Maryland 20892

Received July 15, 1994

Human adenovirus type 2 has been previously shown to increase the delivery of a variety of proteins into cells when Ad is co-internalized with the protein ligands. To increase the efficiency of adenoviral-mediated delivery of proteins, I have linked adenovirus, separately with two proteins, epidermal growth factor and an antibody against human transferrin receptor through disulfide and thioether linkages. Competition experiments indicate that the conjugates are taken up into the cells through adenovirus receptor. During the internalization of adenovirus-protein conjugates into KB cells, the conjugates were equally effective as the native adenovirus in disrupting endocytic vesicles and releasing their protein content into the cytosol. This implies the possible use of adenovirus to deliver a large number of protein molecules into the cells.

In recent years there has been a great interest in developing ways to introduce foreign genes and proteins into eukaryotic cells. One approach which has been successfully used capitalizes on the observation that human adenovirus type 2 or type 5 can mediate the release of proteins, dextrans and DNAs into the cytosol; when these ligands and adenovirus are co-internalized into the cells (1-10). To explain adenovirus-dependent enhancement of protein delivery to cells, it has been suggested that during the entry of adenovirus into cells, when adenovirus escapes from endocytic vesicles into the cytosol, it disrupts the membrane of the endocytic vesicles, thereby releasing the content of the endocytic vesicles (co-internalized protein molecules) into the cytosol (1-3). This process requires the low pH of the endocytic vesicles which makes the penton base of Ad more hydrophobic and activates a endosomal membrane bound Na^+K^+-ATPase, which by an unknown mechanism disrupts the membrane of the endosomes (1, 2, 9-18). Since, this approach of Ad-mediated delivery of proteins requires the process of co-internalization of adenovirus and the protein of interest, it might be of further advantage to try to deliver protein molecules to cells after directly conjugating them with adenovirus.

[1]Present address: Medicine Branch, National Cancer Institute, National Institutes of Health, Bldg. 10, Room 12 C 210, Bethesda, MD 20892. Fax: 301-402-0172.

0006-291X/94 $5.00

Keeping this in mind I have devised ways to conjugate two proteins, epidermal growth factor (EGF) and a monoclonal antibody to the human transferrin receptor, HB21 (19) to adenovirus type2 (Ad). Ad-protein conjugates appear to enter the cells through the same pathway used by Ad indicating that it is feasible to conjugate large amounts of proteins to Ad and hence deliver them to the cells.

MATERIALS AND METHODS

Cells. KB cells (ATCC CCL 17) were grown in Dulbecco's Modified Eagles Medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 50 units/ml penicillin and 100 µg/ml streptomycin (all from Gibco/BRL, Gaithersburg, MD).

Adenovirus. Human adenovirus type2 (Ad) was grown in KB cells, and purified through two CsCl density gradients as described previously (12, 13).

Conjugation of Ad with EGF and HB21. In preliminary experiments it was found that Ad possesses free thiol groups, as shown by its reactivity towards dithionitrobenzoate (DTNB) (Seth and Pastan, unpublished data). The free thiol groups present in Ad were utilized to conjugate two proteins EGF and HB21 through either disulfide or thioether linkages.

For conjugating Ad with EGF by disulfide bonding, a thiol group was introduced in EGF as described previously (3) by treating EGF (0.5 mg containing 3.09 x10^5 cpm of ^{125}I- EGF) with 50 µg of methyl-4-mercaptobutyrimidate . Excess of chemical reagent was removed using a Sephadex G-25 column. Ad (200 µg) and activated EGF were incubated together at room temperature for 2 hr to allow the formation of disulfide bond. The reaction was stopped by the addition of 6 µg of cysteine. Unbound EGF was separated from the Ad-EGF conjugate through a sucrose-glycerol gradient method (12, 13).

For thioether linkage, EGF (0.5 mg containing 2.6 x 10^5 cpm of ^{125}-I EGF) was treated with 50 µg of m-maleimido-benzoyl N-hydroxy succimide at room temperature for 30 min. EGF was separated from the incubation mixture using a Sephadex G-25 column. Ad and activated EGF were mixed at room temperature for 2 h and the Ad-EGF conjugates were separated from free EGF through sucrose-glycerol gradient (12,13).

Similar chemical reactions were used to link HB21 with Ad through disulfide and thioether bonds.

^{35}S- Ad and ^{125}-I EGF binding to cells. ^{35}S-labeled Ad and ^{125}I-EGF binding to KB were measured as described previously (12, 13). In brief, cells were exposed to ^{35}S-Ad or ^{125}I-EGF for 1 hr at 4^{0}C, after which cells were washed with PBS, lysed in 0.1N NaOH and counted for radioactivity.

Delivery of Ad-EGF and Ad-HB21 into cytosol. Release of Ad, Ad-EGF or Ad-HB21 into the cytosol was followed by their ability to enhance the toxicity of a hybrid toxin Pseudomonas exotoxinA (PE) linked with EGF (PE-EGF) or HB21 (PE-HB21 in KB cells using the protocols described previously (12, 13). In brief, cells were exposed to Ad or the Ad-protein conjugate in the presence of toxin conjugate for 1 hr followed by the measurement of ^{3}H-incorporation into host proteins. Percentage of inhibition of protein synthesis (the enhancing activity of Ad or Ad-protein conjugates) was the measurement of the amounts of Ad or Ad-protein conjugates delivered to the cell cytosol.

Purification of fiber protein. Fiber was isolated from the supernatants obtained during the purification of Ad using the procedures described earlier (13, 20) .

RESULTS AND DISCUSSION

Conjugation of Ad with EGF and HB21.

From the specific activities of ^{125}I-Ad-EGF obtained, it was calculated that 663 molecules of EGF were introduced per virus particle. To confirm the chemical linkage through disulfide bonding, the conjugates were analyzed by SDS-gel electrophoresis system in the absence and

presence of reducing agent DTT. When gels were run in the absence of any reducing agent, Ad-EGF conjugate migrated at a position of molecular mass greater than 100,000 kd (Fig.1A), suggesting that most of the EGF was bound to adenovirus capsid proteins. When the conjugates were analyzed in the presence of 10 mM DTT, free EGF dissociated from the conjugate and appeared at its expected position on the gel (Fig.1A). Since, DTT will dissociate any disulfide bonds between the two proteins, it was concluded that Ad is conjugated with EGF through the disulfide bond. It was estimated that 520 EGF molecules were introduced per virus particle.

By using the above described techniques, I was able to conjugate approximately 94 and 129, HB21 monoclonal antibody molecules per virus particle through disulfide and thioether linkages respectively. The former conjugation was confirmed to be through a disulfide bond as the treatment of the conjugates with 10 mM DTT also dissociated free antibody (Fig.1B).

Adenovirus-protein conjugates enter the cells through adenovirus receptor.

In order for Ad-protein conjugates to deliver proteins to cells Ad must enter the cells through Ad receptor and be able to disrupt endocytic vesicles. I therefore investigated if Ad-protein conjugates were entering the cells through Ad receptor or through the EGF receptor (in case of Ad-EGF conjugate) or through the antibody receptor (in case of Ad-HB21 conjugate). For Ad-EGF conjugates (disulfide bonded), I tested the effect of free fiber protein on the ability of the conjugate to enhance the activity of EGF-PE. It was found that the addition of free fiber protein to the incubation mixture inhibited the enhancing effect of the conjugates (Table 1). It has been

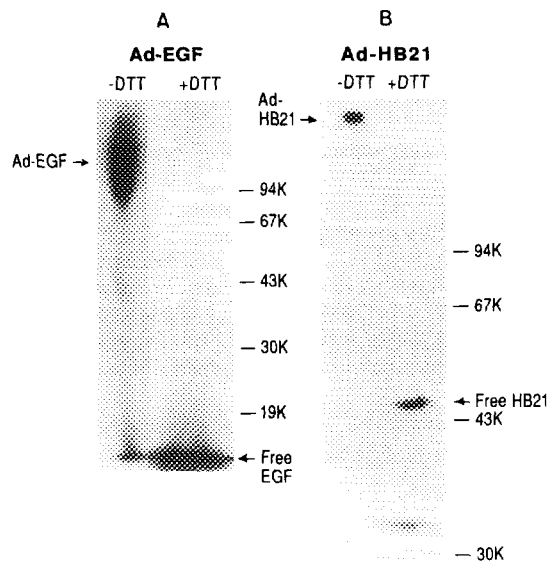

Fig.1. SDS-PAGE of Ad-EGF and Ad-HB21 conjugates. Adenovirus was conjugated separately with ^{125}I-EGF and ^{125}I-HB21 as described in the text. The conjugates were subjected to SDS-PAGE under non-reducing (-DTT) and reducing conditions (+10 mM DTT). After the electrophoresis, the gels were dried and radioautographed as described earlier (13). Gels run in the presence of DTT show the dissociation of EGF and HB21. Molecular weight markers are 94 Kd, phosphorylase b ; 67 Kd, bovine serum albumin; 43 Kd, ovalbumin; 30 Kd, carbonic anhydrase; 19 Kd, soybean trypsin inhibitor.

Table 1

Protein added	Ad-EGF enhancing activity[a] (% control)	^{125}I- EGF binding[b] (% control)	^{35}S-Ad-EGF [c] (% control)
EGF (10 μg/ml)	92	8	81
Fiber (10 μg/ml)	18	94	15

Using KB cells, the ability of Ad-EGF to enhance the effects of EGF-PE (enhancing activity of Ad-EGF) was measured as described in the legend to Fig. 2; the binding of ^{125}I-EGF was studied as described previously (12) and the binding of ^{35}S-Ad-EGF conjugates, as described earlier for ^{35}S-adenovirus (12, 13). Binding experiments were done at 4°C for 1 h. The experiments were conducted in the absence or presence of EGF or fiber. In all the experiments control values represent 100%.

[a] In control cells 92% inhibition of protein synthesis was observed.
[b] CPM bound to control cells were 18358/dish.
[c] CPM bound to control cells were 2612/dish.

previously established that free fiber competes for the binding of Ad to its cell surface receptor (21, 22).On the other hand, excess of free EGF did not block the enhancing effect of the conjugate (Table 1), while under these conditions cold EGF displaced the binding of ^{125}I-EGF to cells (Table 1). Moreover, when Ad-EGF was constructed using ^{35}S-labeled Ad and the resulting conjugate was tested for the binding to KB cells, EGF did not compete for the binding of Ad-EGF conjugate to the cells (Table 1). These results therefore show that the Ad-EGF conjugate does not enter the cells through EGF receptors.

Similar results were obtained when Ad-EGF conjugate made through thioether bonds were used. From these results, I concluded that Ad-EGF enter the cell through the Ad receptor. Similarly, the experiments conducted using Ad-HB21 conjugates have also shown that Ad-HB21 conjugates also enter the cells through adenovirus receptor (data not shown).

Ad-EGF and Ad-HB21 are able to disrupt endocytic vesicles.

If Ad-protein conjugates are to deliver the attached proteins into the cells, the ability of Ad-protein conjugates to escape from the endocytic vesicles should not get destroyed. To test for this, I studied the ability of Ad-protein conjugates to disrupt the endocytic vesicles as measured by their ability to enhance the toxic effects of hybrid toxins. It was found that both Ad-EGF and Ad-HB21 conjugates were able to disrupt the endocytic vesicles with nearly the same efficiency as the unconjugated virus (Fig. 2). Similarly, the conjugates made through thioether linkage were as effective as the native virus (data not shown). These results, therefore, show that conjugating at least two proteins with adenovirus does not alter the lytic activity associated with the virus.

On the basis of the results presented here I believe that Ad-protein conjugates will follow the receptor-mediated endocytosis pathway of adenovirus (1, 2, 23, 24). Since, there are about

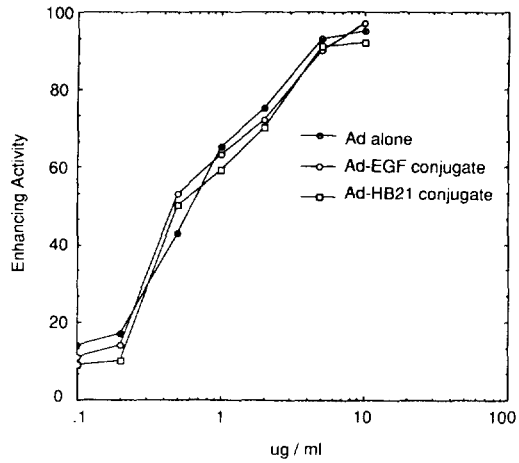

Fig.2. Enhancing activity of Ad-EGF and Ad-HB21. KB cells were used to measure the enhancement of PE-EGF toxicity measured by the inhibition of protein synthesis by the procedure described previously (12, 13). In brief, cells were planted at $5x10^5$ per 35-mm dishes and used 24 h later. Cells were exposed to Ad, Ad-EGF or Ad-HB21 (1 μg/ml each). The enhancing activity of Ad-EGF was tested in the absence and presence of PE-HB21 (0.5 μg/ml), and that of Ad-HB21 in the absence and presence of PE-EGF (0.5 μg/ml) at 37°C for 1h. At the end of the incubation, medium was removed and replaced by 2 ml of DMEM containing ^3H-leucine. The level of protein synthesis was determined by the number of counts per minute in trichloro acetic acid-insoluble material from the cells. Inhibition of protein synthesis is arbitrarily defined as the enhancing activity. Experiments were done in duplicates and counts were averaged. Results shown are the enhancing activity of Ad, Ad-EGF, and Ad-HB21.

10,000 virus receptor per cell (21, 22), and each virus molecule can conjugate at least 100 to 500 molecules of the proteins, it should be possible to introduce about $1\text{-}5x10^6$ copies of the proteins into each cell. During Ad entry into the cytosol, the attached protein will be also released into the cytosol and not be degraded in the lysosomes. Although, so far I have not followed the fate of the adenovirus-mediated proteins in the cells, my future experiments will be directed towards this goal. In addition, it will be interesting to investigate if the free penton base which has been shown to be involved in the lysis of endocytic (13) and membrane vesicles (18) and binding to integrin molecules (25-27) can be also used to deliver proteins to cells.

ACKNOWLEDGMENTS

I am thankful to Ira Pastan and David FitzGerald for their advice and support and to Mario Gallo and Betty Lovelace for technical assistance.

REFERENCES

1. Pastan, I., Seth, P., FitzGerald, D., and Willingham, M. (1986) In A. L. Notkins, and M.B.A. Oldstone (ed.), Concepts in Viral Pathogenesis II, pp. 141-147. Springer-Verlag, New York.

2. Seth, P., FitzGerald, D., Willingham, M., and Pastan, I. (1986) In R. Crowell, K. Lonberg-Holm (ed), pp. 191-195. American Society for Microbiology, Washington, D.C.

3. FitzGerald, D.J.P., Padmanabhan, R., Pastan, I., and Willingham, M.C.(1983) Cell. 32, 607-617.

4. Defer, C., Belin, M.-T., Cailet-Boudin, M.-L., and Boulanger, P. (1990) J. Virol. 64, 3661-3673.

5. Otero, M.J., and Carrasco, L. (1987) Virology. 160, 75-80.

6. Goldmacher, V.S., Blatter, W.A., Lambert, J.M., Mcintyre, G., and Stewart, J. (1989) Mol. Pharm. 36, 818-822.

7. Curiel, D.T., Agarwal, S., Wagner, E., and Cotten, M. (1991) Proc. Natl. Acad. Sci. (USA) 88, 8850-8854.

8. Cotten, M., Wagner, E., Zatloukal, K., and Birnstcil, M. (1993) J. Virol. 67, 3777-3785.

9. Seth, P., Rosenfeld, M., Higginbotham, J., and Crystal, R. (1994) J. Virol. 68, 933-940.

10. Yoshimura, K., Rosenfeld, M.A., Seth, P., and Crystal, R.G. (1993) J. Biol. Chem. 268, 2300-2303.

11. Blumenthal, R., Seth, P., Willingham, M.C., and Pastan, I. (1986) Biochem. 25, 2231-2237.

12. Seth, P., FitzGerald, D.J.P., Willingham, M.C., and Pastan, I. (1984). J. Virol. 51, 650-655.

13. Seth, P., FitzGerald, D., Ginsberg, H. Willingham, M., and Pastan, I. (1984). Mol. Cell. Biol. 4, 1528-1533.

14. Seth, P., Pastan, I., and Willingham, M. (1985) J. Biol. Chem. 260, 9598-9602.

15. Seth, P., Willingham, M.C., and Pastan, I. (1984) J. Biol. Chem. 259, 14350-14353.

16. Seth, P., Willingham, M.C., and Pastan, I. (1985) J. Biol. Chem. 260, 14431-14434.

17. Seth, P., Willingham, M.C., and Pastan, I. (1987) J. Virol. 61, 883-888.

18. Seth, P. (1994) J. Virol. 68, 1204-1206.

19. FitzGerald, D.J.P., Trowbridge, I.S., Pastan, I., and Willingham, M.C. (1983). Proc. Natl. Acad. Sci. (U.S.A.) 80, 4134-4138.

20. Boulanger, P.A., and Puvion, F. (1973) Eur. J. Biochem. 39, 37-42.

21. Ginsberg, H.S. (1988) In Virology 2nd edition. (R. Dulbecco and H.S. Ginsberg Ed.) pp. 147-160. J. B. Lippincot Company, Philadelphia.

22. Horwitz, M.S. (1990) In Fundamental Virology 2nd edition. (B.N.. Fields, D.M. Knipe et al Ed) pp. 1679-1721. Raven Press Ltd., New York.

23. Svensson, U. (1985) J. Virol. 55, 442-449.

24. Grebne, U.F., Willets, M., Webster, P., and Helenius, A. (1993) Cell. 75, 477-486.

25. Wickman, T.J., Mathias, P., Cheresh, D.A., and Nemerow., G.R. (1993) Cell. 73, 309-320.

26. Bai, M., Harfe, B., and Freimuth, P. (1993) J. Virol. 67, 5198-5205.

27. Belin, M-T, and Boulanger, P. (1994) J. Gen. Virol. 74, 1485-1497.