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Altered Expression of Fibronectin Gene in Cells Infected with Human Cytomegalovirus

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Human cytomegalovirus (HCMV) induces morphological changes in infected cells that are remarkably similar to those seen in oncogenically transformed cells. The molecular bases of these phenotypic alterations are not known but their occurrence in some transformed cells can be associated with abnormal fibronectin (FN) expression. In this report, we have compared FN levels in normal and HCMV-infected cells. In these studies, the HCMV-infected fibroblasts exhibited a progressive loss of cellular FN. Northern (RNA) blot analysis revealed that the decrease in FN levels resulted from a lowering of FN mRNA levels in HCMV-infected cells. We detected an initial decrease in FN mRNA of 25 to 30% at immediate-early and early times, whereas at late times after infection the levels of FN mRNA were lowered by >80%. These results indicated that the HCMV-induced decrease in FN expression is due to a decrease in the quantity of FN mRNA and suggested that HCMV-encoded and/or -induced functions may be involved in producing these alterations.

The ability of some viruses to alter the "luxury" or differentiated cellular functions without killing the host is well recognized (17, 18). Examples of this effect include growth hormone inhibition by lymphocytic choriomeningitis virus (19) and altered immunoglobulin synthesis by measles and influenza viruses (5). Similarly, oncogenic viruses can alter the level of a number of extracellular matrix components, including fibronectin (FN), collagen, and laminin. Loss of FN, in particular, is related to reduced adhesion and altered morphology of virus-transformed cells, since addition of exogenous FN can revert these phenotypic properties in many cases (2, 23).

One striking aspect of the phenotype of cells infected with human cytomegalovirus (HCMV) is the rounded shape with reduced adhesiveness that typifies the characteristic cytopathic effect (1). It is likely that these morphological changes are, in some respects, similar to those seen in virus-transformed cells and may be related to FN expression. Since earlier reports describing FN analysis of HCMV-infected cells have provided conflicting results (13, 21), we have attempted to analyze levels of FN in normal and HCMV-infected cells. We have used domain-specific monoclonal antibodies (MAbs) to FN and mRNA analysis to investigate FN expression in normal and HCMV-infected cells. In these studies, HCMV-infected fibroblasts exhibited a progressive loss of cellular FN. Furthermore, the decreased amount of FN correlated with the decreased levels of FN mRNA.

HCMV (Towne strain) was grown in human foreskin fibroblast (HFF) monolayer cultures and harvested for antigen production at 4 days postinfection (p.i.). The cells (approximately 2×10^7) were quantitatively removed from each flask and solubilized in 1 ml of 10× sample buffer (100 mM Tris hydrochloride [pH 6.8], 5% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol, 20% glycerol) for SDS-polyacrylamide gel electrophoresis. The antigen (25 μl per sample) prepared in this way from normal and HCMV-infected HFF cells was electrophoresed on an SDS–6% polyacrylamide slab gel and electrophoretically transferred to nitrocellulose membranes. The FN band was visualized either by direct staining with Coomassie blue or by immunostaining with domain-specific MAbs to human FN. MAbs IST-2 and IST-9 were employed to examine whether there are unique structural differences between FN produced from normal and HCMV-infected cells. MAb IST-2 is specific for all FN isoforms (6), whereas IST-9 is specific for an FN fragment (ED-A) that can be included or omitted from the molecule depending on the pattern of mRNA splicing (3, 4). A schematic representation of the domain structure of human FN and the localization of epitopes recognized by the two MAbs are shown (Fig. 1). The ED-A segment has been reported to be absent in plasma FN (15, 20), but in transformed cells the ED-A-containing FN is present at a significantly higher level compared with that in normal cells (3). When HCMV-infected cells were analyzed by immunoblot analysis using MAbs and compared with normal HFF cells (Fig. 2), there were significantly reduced amounts of a protein band of 240 to 250 kilodaltons, which immunostained with either MAb IST-2 or IST-9. This decrease observed on the immunoblot appeared to be similar in magnitude to the changes found in the FN band obtained by staining with Coomassie blue (Fig. 2).

To determine whether the decreased amount of FN correlated with reduced levels of FN mRNA in HCMV-infected cells, we studied the steady-state levels of FN mRNA in normal and HCMV-infected cells by Northern (RNA blot) analysis. Since it is possible that FN produced by HCMV-infected cells might be structurally distinct, because of a modification in the splicing of FN pre-mRNA precursors (11), we used several unique DNA probes for RNA blot hybridization. The probes used for this analysis included a 1,300-base-pair (bp) cDNA fragment corresponding to the carboxy-terminal region of FN. This cDNA fragment was isolated from a human liver cDNA library cloned in λgt11 vector and is common to all FN isoforms. Additionally, we used two oligonucleotide probes (ED-45-mer and IIICS-45-mer) that correspond to two distinct alternatively spliced

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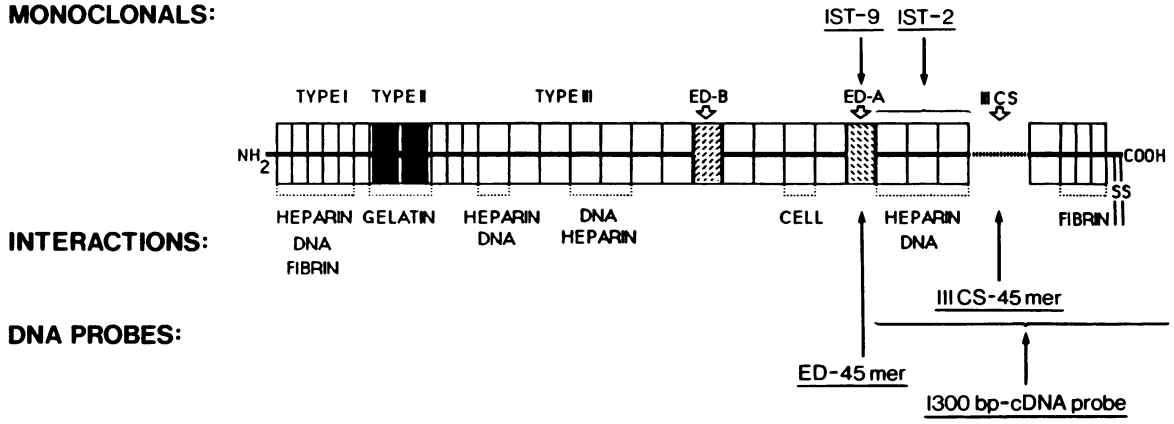

FIG. 1. Schematic representation of the structure of a subunit of human FN. ED-A, ED-B, and IIICS represent the three known regions of variability due to alternative splicing of mRNA precursors. The sites of specificity of the two MAbs used are indicated. The map locations of the two oligonucleotides (ED-45-mer and IIICS-45-mer) and a cDNA probe are also indicated. The nucleotide sequences of ED-45-mer and IIICS-45-mer are 5'- CATATCATCGTGCAAGGCAACCACACTGACTGTGTACTCAGAACC-3' and 5'-GCTGACCAGAAGTGCCAGGA AGCTGAATACCATTTCCAGTGTCAT-3', respectively. The three types of internal homology within the FN molecule are also depicted.

regions (ED-A and IIICS) of FN. The sequence and localization of these probes are shown in Fig. 1. Both oligonucleotide probes were checked for sequence homology to other regions of the FN molecule and appeared to be unique to these regions in FN.

Total RNA was prepared (8) from normal and HCMV-infected HFF cells (multiplicity of infection, 0.4) at 10 days postinfection, when viral cytopathic effect became evident for ~100% of the infected cell monolayer. Samples containing 20 μg of total RNA were resolved by agarose gel electrophoresis, transferred to GeneTrans-45 (Plasco) and probed for FN mRNA. As shown in Fig. 3, HCMV-infected cells had significantly decreased levels of FN mRNA. This decrease was detected by all three FN probes. A densitometric quantitation of the autoradiogram (Fig. 3A through C) showed that the amount of FN mRNA present in HFF cells late after HCMV infection was in the range of 17 to 20% of normal. To determine whether this observation was specific for FN, the same blot was stripped of the FN probe and reprobed with a 2.0-kilobase actin cDNA probe. The amount of actin mRNA in HCMV-infected HFF cells was found, by densitometry, to be ~95% of that found in normal cells.

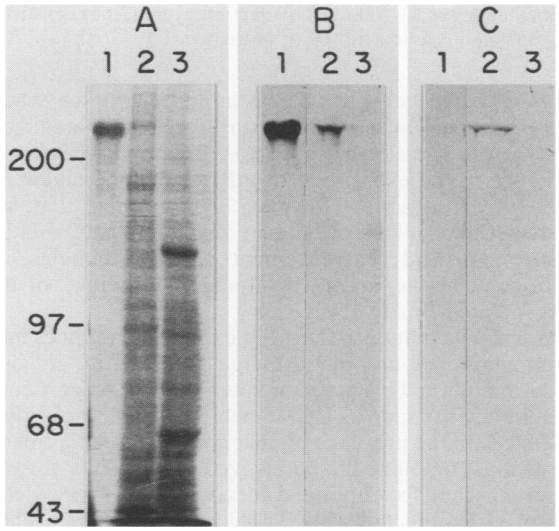

FIG. 2. SDS-polyacrylamide gel electrophoresis and immunoblot analyses of total cell lysates from normal and HCMV-infected HFF cells. Purified plasma FN (lanes 1) and total lysates from 5×10^5 HFF cells (lanes 2) or from the same number of HFF cells that were infected with HCMV for 4 days (lanes 3) were analyzed on an SDS–6% polyacrylamide gel followed by staining with Coomassie blue (A) or by immunostaining using either MAb IST-2 (B), which recognizes all isoforms of FN, or MAb IST-9 (C), which is specific for the ED-A region present in cellular FN but not in plasma FN. Levels of FN appear to be significantly reduced in HCMV-infected cells compared with those of their normal counterparts.

The data presented above clearly demonstrate that at late times after HCMV infection there is a significant decrease in FN levels in the infected cells and that this is due to a decrease in the amount of translatable FN mRNA. We next investigated whether the suppression of FN gene expression was temporally associated with the expression of one or more HCMV-encoded gene products. Since the synthesis of each of the HCMV immediate-early (IE), early, and late gene classes is coordinately regulated (22), it was of interest to examine the levels of FN mRNA at these intervals.

HCMV IE RNA was generated by treating cells with 50 μg of cycloheximide per ml at 1 h before infection. At 5 h p.i., cells were treated with dactinomycin (5 μg/ml) to inhibit further transcription of viral DNA. The cycloheximide block was removed at 6 h p.i. to allow the translation of IE mRNA and cells were harvested 5 h later (11 h p.i.). To accumulate early transcripts, cells were treated with phosphonoacetic acid (100 μg/ml) at 2 h p.i. and harvested 28 h later (30 h p.i.). For late RNA, infected cells were harvested at 30 h p.i. in the absence of any inhibitors. Samples containing 12 μg of total RNA corresponding to IE, early, or late RNA species were analyzed by agarose gel electrophoresis, transferred to GeneTrans-45 membrane and probed with the 1,300-bp FN cDNA probe (Fig. 4). A densitometric quantitation of the Northern blot demonstrated a decrease of approximately 25 to 30% in FN mRNA levels at IE times after HCMV infection. At early times after HCMV infection, we did not observe a significant reduction in FN mRNA levels compared with those observed at IE times. However, at late times after HCMV infection there was a marked decrease (>80%) in FN mRNA levels. This observation is in agreement with previous reports that virus infection decreases

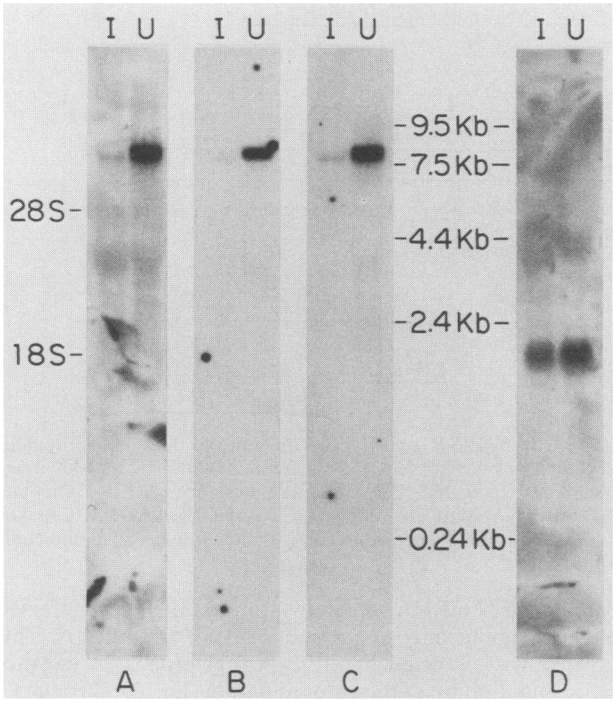

FIG. 3. Northern blot analysis to investigate the effect of HCMV infection on levels of FN mRNA. Total RNA (20 μg) from normal HFF cells (lanes U) or from HCMV-infected HFF cells (multiplicity of infection, 0.4) at 10 days p.i. (lanes I) were applied to each lane and fractionated by electrophoresis on a 1% agarose gel containing formaldehyde. The gel was blotted onto GeneTrans-45 membrane and probed with a ^{32}P-labeled 1,300-bp cDNA fragment corresponding to the C-terminal region of FN (A), ED-45-mer, a 45-base-long oligonucleotide probe corresponding to the ED-A region of FN (B), IIICS-45-mer, a 45-base-long oligonucleotide probe corresponding to the IIICS region of FN (C), or a 2-kilobase cDNA probe for actin (D).

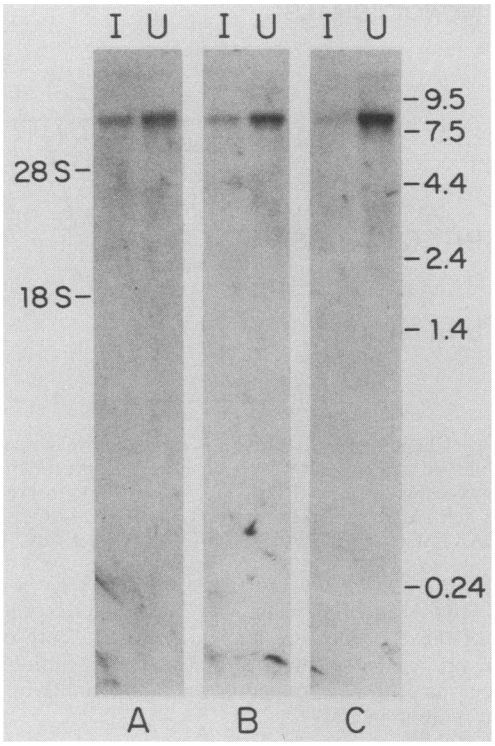

FIG. 4. Northern blot analyses to investigate the effect of IE, early, or late gene functions of HCMV on levels of FN mRNA. Whole-cell RNA (12 μg) from uninfected HFF cells (lanes U) or HCMV (Towne)-infected HFF cells (lanes I) were applied to each lane and fractionated by electrophoresis on a 1% agarose gel containing formaldehyde. The gel was blotted onto GeneTrans-45 and probed with a ^{32}P-labeled 1,300-bp cDNA probe for FN. The HCMV-infected cells included cells that had been treated with cycloheximide for 5 h followed by dactinomycin (A) or cells treated with phosphonoacetic acid (B) or untreated (C).

host cell protein synthesis at late times after infection. The decrease at IE times, however, was not expected and suggests that multiple HCMV-encoded and/or -induced factors are involved in this phenomenon.

We have demonstrated evidence that HCMV infection alters expression of FN, a protein which may be considered as an example of a luxury cell function. Disrupted synthesis of FN does not adversely affect the survival of a cell; however, it may jeopardize the maintenance of topological and immunological homeostasis. FN binds complement components and may facilitate the clearance of immune complexes by phagocytosis. In addition, FN binds to several microbial antigens, including bacterial, protozoal, and viral structures, and may enhance phagocytosis by functioning as an opsonin (14, 16). Thus, decreased FN synthesis may alter host antibody-mediated cellular defenses.

FN was originally discovered as a protein missing from the surfaces of virus-transformed cells. Subsequently, the loss of extracellular FN in fibroblasts infected with hamster sarcoma virus (12), polyomavirus (9, 12), simian virus 40 (10), and adenovirus (7) has been demonstrated with cell surface labeling and immunochemical techniques. Since loss of FN correlates with changes in cell morphology rather than tumorigenic properties of virus-transformed cells, we propose that this alteration is, at least in part, a molecular explanation for the morphological changes observed for HCMV-infected cells. It will be important to see whether other proteins, which are necessary for the maintenance of cell structure, are altered by HCMV infection. We observed no change in the expression of mRNA for one major cytoskeletal protein, actin. However, it is possible that, as in transformed cells, lack of FN may result in posttranslational redistribution and rearrangement of actin bundles, thus contributing to the observed altered morphology of fibroblasts infected by HCMV.

It is possible that decreased FN expression reduces infected-cell adherence and could increase spread by enhancing cellular mobility. This mode of spread would be particularly useful for a virus such as HCMV which is predominantly cell associated. In the presence of host immunity, cell-associated viruses must mobilize the parasitized cell in order to spread and/or to escape for continued survival. In this way, detachment of virus-infected cells would not merely be a change due to imminent cell death but a means for the virus to spread to other cells or to move away from a localized and activated immune attack.

The identity of a particular HCMV function(s) which may be involved in altering FN gene expression is not known and deserves further investigation. A number of HCMV genes have now been cloned and characterized. These should provide useful tools to further an understanding of the molecular basis for the altered morphology and cytoskeletal disorganization seen in HCMV-infected cells.

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LITERATURE CITED

1. **Albrecht, T., T. Cavallo, N. L. Cole, and K. Graves.** 1980. Cytomegalovirus: development and progression of cytopathic effects in human cell culture. Lab. Invest. **42**:1–7.
2. **Ali, I. U., V. Mautner, R. Lanza, and R. O. Hynes.** 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. Cell **11**:115–126.
3. **Borsi, L., B. Carnemolla, P. Castellani, C. Rosellini, D. Vecchio, G. Allemanni, S. E. Chang, J. Taylor-Papadimitriou, H. Pande, and L. Zardi.** 1987. Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. J. Cell Biol. **104**:595–600.
4. **Carnemolla, B., L. Borsi, L. Zardi, R. J. Owens, and F. E. Baralle.** 1987. Localization of the cellular-fibronectin-specific epitope recognized by the monoclonal antibody IST-9 using fusion proteins expressed in *E. coli*. FEBS Lett. **215**:269–273.
5. **Casali, P., G. P. A. Rice, and M. B. A. Oldstone.** 1984. Viruses disrupt functions of human lymphocytes. J. Exp. Med. **159**: 1322–1337.
6. **Castellani, P., A. Siri, C. Rosellini, E. Infusini, L. Borsi, and L. Zardi.** 1986. Transformed human cells release different fibronectin variants than do normal cells. J. Cell Biol. **103**: 1671–1677.
7. **Chen, L. B., P. H. Gallimore, and J. K. McDougall.** 1976. Correlation between tumor induction and the large external transformation sensitive protein on the cell surface. Proc. Natl. Acad. Sci. USA **73**:3570–3574.
8. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
9. **Gahmberg, C. G., D. Kiehn, and S.-I. Hakomori.** 1974. Changes in a cell-surface labelled galactoprotein and glycolipid concentrations in cells transformed by a temperature sensitive polyoma virus mutant. Nature (London) **248**:413–415.
10. **Hogg, N. M.** 1974. A comparison of membrane proteins of normal and transformed cells by lactoperoxidase labeling. Proc. Natl. Acad. Sci. USA **71**:489–492.
11. **Hynes, R.** 1985. Molecular biology of fibronectin. Annu. Rev. Cell Biol. **1**:67–90.
12. **Hynes, R. O.** 1973. Alterations of cell-surface proteins by viral transformation and proteolysis. Proc. Natl. Acad. Sci. USA **70**:3170–3174.
13. **Ihara, S., A. Saito, and Y. Watanabe.** 1982. Suppression of fibronectin synthesis by an early function(s) of human cytomegalovirus. J. Gen. Virol. **59**:409–413.
14. **Keski-Oja, J., A. Hautanen, and I. Julkunen.** 1987. Fibronectin and viral pathogenesis. Rev. Infect. Dis. **9**:S404–S411.
15. **Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle.** 1984. Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. EMBO J. **3**:221–226.
16. **Mosher, D. F.** 1980. Fibronectin. Prog. Hemostasis Thromb. **5**:111–151.
17. **Oldstone, M. B. A.** 1984. Virus can alter cell function without causing cell pathology: disordered function leads to imbalance of homeostasis and disease, p. 269–276. *In* A. B. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
18. **Oldstone, M. B. A.** 1989. Viral alteration of cell function. Sci. Am. **216**:42–48.
19. **Oldstone, M. B. A., Y. N. Sinha, P. Blount, A. Tishon, M. Rodriguez, R. von Wedel, and P. W. Lampert.** 1982. Virus-induced alterations in homeostasis: alterations in differentiated functions of infected cells in vivo. Science **218**:1125–1127.
20. **Pande, H., J. Calaycay, T. D. Lee, K. Legesse, J. E. Shively, A. Siri, L. Borsi, and L. Zardi.** 1987. Demonstration of structural differences between the two subunits of human-plasma fibronectin in the carboxy-terminal heparin-binding domain. Eur. J. Biochem. **162**:403–411.
21. **Radsak, K., I. Mertensmaier, C. Kaiser, and C. Wagner.** 1985. Distinction of viral and host-derived glycopolypeptides induced by "early" functions of human cytomegalovirus. Arch. Virol. **85**:217–230.
22. **Stinski, M. F.** 1983. The molecular biology of cytomegaloviruses, p. 67–113. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Plenum Publishing Corp., New York.
23. **Yamada, K. M., S. S. Yamada, and I. Pastan.** 1976. Cell surface protein partially restores morphology, adhesiveness, and contact inhibition of movement to transformed fibroblasts. Proc. Natl. Acad. Sci. USA **73**:1217–1221.