GRADUATE PROGRAM IN

THE MOLECULAR BASIS OF HUMAN DISEASE

" The effect of TGF-β **growth factor** on Herpes Simplex Virus type 1 (HSV-1) progeny viral yield during lytic infection "

Molecular Basis Of Human Diseases

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« Η επίδραση του αυξητικού παράγοντα TGF-β στην παραγωγή ιικών σωματιδίων κατά την διάρκεια λυτικής λοίμωξης με τον ιό του απλού έρπητα τύπου 1 (HSV-1)»

Μοριακή Βάση των ασθενειών του ανθρώπου
Πανεπιστήμιο Κρήτης, Ιατρική σχολή, 2007-2008
Επιτροπή αξιολόγησης
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ΠΕΡΙΛΗΨΗ

Όπως όλα τα ενδοκυττάρια παθογόνα (ιοί, βακτήρια, παράσιτα), έτσι και ο ιός του απλού έρπητα τύπου ένα έχει προσαρμοστεί στην εξελικτική πίεση ώστε να χρησιμοποιεί κυτταρικές πρωτεΐνες και να χειραγωγεί ενδοκυττάρια μονοπάτια προς όφελός του. Το ιικό γονιδίωμα κωδικοποιεί για περιορισμένο αριθμό πρωτεϊνών που δεν επαρκούν για την εξασφάλιση της αποτελεσματικής μεταγραφής του, ακόμα και για την περίπτωση των μεγαλύτερων DNA ιών. Η χρήση πληθώρας κυτταρικών πρωτεϊνών από τον ιο του έρπητα εγγυάται τον πολλαπλασιασμό των ιικών σωματιδίων, ενώ ο επαναπρογραματισμός των ενδοκυττάριων σηματοδοτικών μονοπατιών μεταβάλλει σημαντικές λειτουργίες του κυττάρου όπως η ανοσολογική απόκριση, ο αποπτωτικός θάνατος κλπ.

Η ισορροπία μεταξύ του κυττάρου ξενιστή και του ιού επιτυγχάνεται μέσω πολύπλοκων μηχανισμών που καταλήγουν σε αλλαγές στην έκφραση ποικίλων γονιδίων του κυττάρου. Το πρότυπο των αλλαγών αυτών αλλάζει σημαντικά κατά την διάρκεια της παραγωγικής λοίμωξης από τον ιό του έρπητα, σε σχέση με την λανθάνουσα κατάσταση του ιικού γονιδιώματος στους νευρώνες. Η χρήση μικροσυστιχιών DNA τα τελευταία χρόνια επιτρέπει την δυνατότητα μελέτης των αλλαγών στην έκφραση πληθώρας κυτταρικών γονιδίων ως απόκριση σε ποικιλία ερεθισμάτων και συνθηκών. Χρησιμοποιώντας την τεχνική αυτή, πρόσφατες δημοσιεύσεις μελέτησαν την αλλαγή στο πρότυπο γονιδιακής έκφρασης κυττάρων στο πλαίσιο λυτικής λοίμωξης από τον ιό του έρπητα τύπου 1. Μεταξύ των πολλών γονιδίων που παρουσίασαν αλλαγές ως απόκριση στην παρουσία του ιού, ήταν και γονίδια που εμπλέκονται στο σηματοδοτικό μονοπάτι του TGF-β. Ποιο συγκεκριμένα η παρουσία του ιού οδήγησε σε μειωμένη έκφραση γονιδίων που κωδικοποιούν για πρωτεΐνες του μονοπατιού του TGF-β. Ωστόσο μέχρις στιγμής, δεν υπάρχουν μελέτες που να καταδεικνύουν την επίδραση του παράγοντα αυτού στην παραγωγή προγονικών ιικών σωματιδίων στο πλαίσιο λυτικής λοίμωξης.

Στην παρούσα μελέτη εξετάσαμε την επίδραση του TGF-β στην παραγωγή προγονικών ιών κατά τη διάρκεια της λυτικής λοίμωξης με τον ιό του απλού έρπητα τύπου 1 (HSV-1). Βασισμένοι στα ανωτέρω δεδομένα, περιμένουμε να δούμε μείωση στην απελευθέρωση ιικών σωματιδίων κατά τη διάρκεια διέγερσης με TGF-β.

SUMMARY

Alike many intracellular pathogens (viruses, bacteria, parasites), Herpes Simplex Virus type 1 (HSV-1) has evolutionary evolved throughout the course of its existence to employ cellular proteins and modulate cellular signalling cascades for its own purposes. Viral genome in general encodes for a limited number of proteins not sufficient to satisfy viral functions even for the largest DNA viruses. HSV-1 has adjusted to utilize cellular proteins to guaranty its replication, and manipulate several signalling pathways to modulate cellular functions such as immune response, apoptosis e.t.c.

The balance between host cells - virus interactions is achieved by complex mechanisms resulting in complete re-organization of the expression pattern of a great number of cellular genes. This pattern fundamentally changes when HSV-1 undergoes lytic infection compared to HSV-1 established latency in neuronal cells. DNA microarray studies have provided us with a useful tool for highthroughput analysis of the expression pattern of a wide variety of cellular genes upon diverse conditions. Using this technique, recent publications studied modulated gene expression upon HSV-1 productive infection. Among the many different signalling cascades shown to be altered, the TGF-β pathway was one of them. More specifically, HSV-1 lytic infection down regulated proteins implicated in TGF-β signaling cascade. However, until so far there has been no report of the actual effect of TGF-β stimulation on the release of progeny viruses during HSV-1 productive infection.

In this study we used in vitro cell cultures to examine the release of HSV-1 progeny viruses upon TGF-β stimulation. We used 2 different HSV-1 viral strains. HSV-1 17+ (laboratory wild-type strain) and its fluorescent counterpart VECFP-ICP4. Based on the data presented above, if our hypothesis is correct we expect to see abrogated virion release upon TGF-β stimulation.

CONTENTS

INTRODUCTION

1. The Herpesviridae family

Herpesviruses are large double stranded DNA viruses, highly disseminated in nature. Since they naturally infect more than one species, the number of herpesviruses in nature probably exceeds 200. Thus far, 8 viruses from this family have been identified to have humans as their primary hosts: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and Human herpesviruses 6, 7 and 8 (HHV-6, HHV-7, and HHV-8 or Kaposi's sarcoma herpesvirus) (1,2).

Each of these viruses possesses unique characteristics in terms of primary and latent infections, and host cell-pathogen interactions. However, they share significant aspects of their biological properties.

Heprpesviruses specify a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g. DNA polymerase, helicase, primase) and processing of proteins (e.g., protein kinases), although the exact array of enzymes may vary from one herpesvirus to another. The synthesis of viral DNAs and capsid assembly occurs in the nucleus. Final processing of the virion takes place in the cytoplasm followed by release of viral progeny. Production of infectious progeny virus is accompanied by the destruction of the infected cell, an effect called cytopathic effect. Finally, their most significant biological property that also named the family, is their ability to achieve life-time latent infections in humans. The word hepresvirus derives from the Greek word herpein which means to crawl, describing the ability of these viruses to recurrence from latency and produce lytic infections. All herpesviruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes is expressed. Latent genomes retain the capacity to replicate and cause disease on reactivation. The precise molecular mechanisms that lead to reactivation from the latent state are not fully understood and may differ from one virus to another. Latency differs from chronic

infection in that infectious progeny are not present. Conversely, the capacity to reactivate differentiates latency from abortive infection. A virus can be simultaneously latent in some cells and actively proliferating in others. Thus, there are circumstance when (a) virus is latent in essentially all infected cells (as seems to be the case for VZV and, to a lesser extent, for HSV); (b) virus is lytically active in a subset of infected cells, but there are no associated symptoms (asymptomatic shedding of HSV—the most common state of affairs for all of the viruses but perhaps VZV); and (c) lytic virus activity is of a nature that results in illness (with some cells remaining in a latent state) (1,2).

Despite the similarities in their biological properties, Herpesviruses also differ in many aspects. Some have a wide host cell range, multiply efficiently, and rapidly destroy the cells they infect (e.g., HSV-1, HSV-2). Others have a narrow host cell range (EBV, HHV-6) or a long replicative cycle (HCMV). Although all herpesviruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one virus to another. These differences allow further classification to 3 categories; α- herpesviruses, β- herpesviruses and lastly γ-herpesviruses (3,4).

The α herpesviruses, herpes simplex virus types 1 and 2, and varicella-zoster virus, have a short replicative cycle, induce cytopathology in monolayer cell cultures, and have a broad host range; β herpesviruses, cytomegalovirus, and human herpesviruses 6 and 7, with a long replicative cycle and restricted host range; and γ herpesviruses, Epstein-Barr virus and human herpesvirus 8, with a very restricted host range. Based on their tropism regarding latent infections they are further classified into neurotropic (HSV-1, HSV-2 and VZV) and lymphotropic (EBV, HCMV, HHV-6, HHV-7 and HHV-8) (5). Table.1.

	Virus	Primary infection	Immunosuppresed hosts	Associated malignancies
NEUROTROPIC	HSV-1	•Gingivostomatitis •Encephalitis •Genital lesions	•Oesophagitis •Pneumonitis •Hepatitis •Disseminated gingivostomatitis	None
	HSV-2	•Genital lesions •Ginginostomatitis •Neonatal herpetic infection	•Genital lesions •Disseminated infections	None
	VZV	•Chickenpox (primary) •Herpes zoster (reactivation)	Disseminated infections	None
LYMPHOTROPIC	HCMV	•Congenital infections •Mononucleosis	•Retinitis •Hepatitis •Colitis •Pneumonitis	None
	HHV-6	Roseola infantum	?	?
	HHV-7	?	None	None
	EBV	Mononucleosis	•Lymphoproliferative disorders •Oral hairy leukoplakia	•Burkitts lymphoma •CNS lymphoma •Nasopharyngeal carcinoma
	HHV-8 (KSHV)	?	Kaposi's sarcoma	•Kaposi's sarcoma •Multicentric Castlemans disease •Primary effusion lymphoma

Table.1. Table 1 summarizes the classification of the human herpesviruses, their clinical manifestations during primary infections and their association with virally induced human malignancies.

1.1. Herpes Simplex Virus Type 1 (HSV-1)

1.1.1. Primary and recurrent Infections

Primary herpesvirus infections in the immunocompetent host are usually asymptomatic or are associated with a minor illness only, with no specific symptoms such as fever and malaise. As a consequence, primary herpesvirus infections may not be recognized. When symptoms do occur, herpesvirus infections in the immunocompetent host are normally self-limiting and require only symptomatic treatment. Antiviral therapy is available for a number of herpesviruses, but is usually only indicated for those patients who have more severe disease manifestations or when it is appropriate to minimize the likelihood of complications (6).

Although HSV infections can result in a wide spectrum of disease, mucocutaneous lesions are by far the most common manifestation of primary HSV infections (3).

Primary oropharyngeal HSV-1 infection usually occurs in childhood and is often asymptomatic. In symptomatic infections acute gingivostomatitis is seen in 10–30% of cases. Lesions on the buccal and gingival mucosa evolve from vesicles to shallow ulcerations. Primary HSV infection in an adolescent or adult is often associated with pharyngitis and a mononucleosis-like syndrome (3).

The severity and duration of clinical illness in recurrent infection is considerably less than in primary HSV infection. Recurrent lesions ('cold sores') are heralded by a prodrome of pain, burning, itching several hours before the development of the characteristic vesicles. Precipitating factors involved in HSV recurrence are not well defined, but include the type of HSV (HSV-1 is more likely to recur than HSV-2), fever, stress, exposure to ultraviolet light and impaired cell mediated immunity in the host (4). HSV-1 can also be the cause of life threating diseases such as herpetic encephalitis especially in neonates. It is also the first cause of blindness by infectious agent in the USA due to recurrent infections causing keratoconjunctivitis. In immunocompromised hosts HSV-1 can be the cause of disseminated hepretic lesions complicated by hepatitis, pneumonitis and meningitis often leading to death. Drug resistant viral strains isolated especially from HIV positive patients, are of extreme importance in clinical practice (7,8). Resistant HSV infections in immunosuppressed patients can be the cause of

significant mortality, highlighting the need for further studies in HSV biology and drug development.

1.1.2. Herpes Simplex Virus-1 structure

The herpesvirus virion often has a pleomorphic appearance when seen by electron microscopy. It measures 150–300nm in diameter and is composed of an internal protein nucleocapsid enclosing the double-stranded (ds) DNA genome and an external lipid envelope. The electron microscopic appearance of a typical herpesvirus is shown in Fig.1.

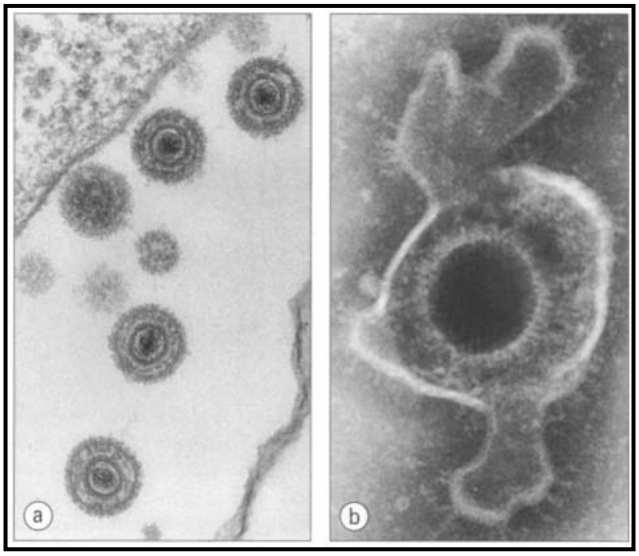


Fig.1. Photograph from electron microscopy. A) Thin section, B) DNA is surrounded by a nucleocapsid comprised of 162 individual protein subunits (150 hexavalent capsomers and 12 pentavalent capsomers) arranged in the form of an icosahedron. The nucleocapsid is in turn enclosed by the tegument and virus envelope bearing glycoprotein spikes. (Picture courtesy of Hans Gelderblom).

Viral DNA; The genetic information of the virus is encoded by a linear molecule of dsDNA and the size of this molecule varies for different herpesviruses, from

approximately 80,000 to 150,000kDa (125–245kbp).Within the virion the DNA is a linear molecule with its ends appearing to attach to the inner surface of the nucleocapsid, thus preventing DNA circularization until it is released during infection.

Virion polypeptides; Herpes simplex virus type 1 virions contain about 33 virus-specific proteins. The virion polypeptides (VPs) are designated by serial number. The transcription of mRNAs from the genome proceeds from both strands of the genome, in either direction, with evidence of overlapping transcription and of splicing of genes and gene products. There are two sets of *cis*-acting genes embedded in the domains of viral genes. The first set enables binding of cellular transcription factors and *trans*-acting factors to initiate and enhance viral gene expression (2). The second set enables interaction of genes with regulatory proteins (up- or downregulation). Three rounds of transcription and translation are observed, the so-called:

α- Phase; Resulting in the production of Immediate Early protein (IE proteins).

β- Phase; Proteins responsible for DNA replication.

γ- Phase; Proteins implicated in structural process and release of progeny viruses.

Nucleocapsid; The icosahedral nucleocapsid is 100–110nm in diameter comprising 162 individual capsomers (i.e. 12 pentavalent and 150 hexavalent capsomers). In HSV individual capsomers are believed to be constructed from the four major capsid proteins VP5, VP26, VP23 and VP19. The interior and exterior of the capsid appear to be linked by transcapsomeric channels (2).

Tegument; The amorphous electron-dense tegument bounded by the nucleocapsid and outer envelope of the virus, contains at least eight proteins. These proteins serve important functions after the virus has penetrated the host cell (2).

Envelope; The viral envelope is an extensively modified form of the original host cell nuclear membrane and bears a series of virus-specified glycoproteins. These proteins define several of the major biologic attributes of the virus. In HSV there are at least ten glycoproteins on the viral envelope (2).

1.1.3. HSV-1 replication cycle

1.1.3.1 Viral gene transcription during lytic HSV-1 infection

The life cycle of HSV involves both lytic (productive) and latent (non-productive) infection. Upon entry at a mucosal surface, or at a break in the skin, HSV infects epithelial cells and undergoes a productive infection (2). Entry involves binding of the virion to the cell surface, which is followed by fusion of the virion envelope and the cell plasma membrane. The viral nucleocapsid is transported along microtubules and then docks with the nuclear pores to release the viral genome into the nucleus. The linear viral DNA circularizes rapidly and is transcribed to sequentially express immediate–early (IE), early (E) and late (L) viral gene products. The nucleus is reorganized to form replication compartments in which viral DNA is replicated and transcribed and progeny nucleocapsids are assembled. The nucleocapsids acquire tegument proteins and an envelope during budding through the inner nuclear membrane. Extracellular virions are produced by de-envelopment of the nucleocapsids at the outer nuclear membrane, which is followed by budding into the Golgi apparatus and secretion to the outside of the cell. Progeny viruses can infect surrounding cells and cause either primary herpetic disease or an asymptomatic infection. **Fig.2.**

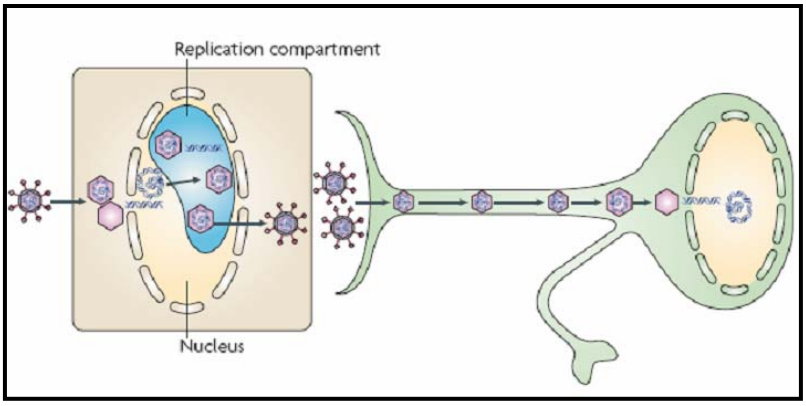


Fig.2. Schematic representation of the release of HSV-1 progeny viruses from infected epithelial cells. Lytic infection leads to destruction of the cells allowing progeny viruses to enter the axon of sensory neurons. The capsid is then transported along microtubules to the neuronal nucleus, establishing viral latency.
Picture courtesy of David M. Knipe and Anna Clife *(Nature 2008).*

During lytic infection, more than 80 viral genes (2) are expressed in a cascade pattern (9). IE gene products are expressed from 2–4 hours post infection. These include infected cell protein 0 (ICP0), ICP4, ICP22, ICP27 and ICP47. The genes that encode these proteins are transcribed in the absence of *de novo* viral gene expression. IE gene promoters have several binding sites for cellular transcription factors, and their transcription is also activated by the virion protein VP16. VP16 is a well characterized transcriptional activator protein. VP16 forms a complex with host cell factor (HCF) and localizes in the cell nucleus, where the VP16–HCF complex binds to the host transcription factor octamer-binding protein 1 (OCT1). OCT1 binds to specific sites in the upstream regulatory sequences of IE genes, tethering the VP16–HCF complex to IE gene promoters and enabling VP16 to recruit transcription factors that stimulate IE gene transcription. IE gene products activate expression of the E gene products. ICP4 is required for all subsequent viral gene expression, most likely through its association with transcription factors (10,11). The E gene products are involved in viral DNA replication, after which the L genes are expressed. Activation of L gene expression requires DNA synthesis and at least 3 viral proteins: ICP4, ICP27 and ICP8. ICP27 reportedly stimulates transcription of L genes, cytoplasmic transport of viral mRNAs and translation of L mRNAs, but the mechanism that underlies these functions has not been fully defined (2). ICP8 probably has a role in viral chromatin modulation.

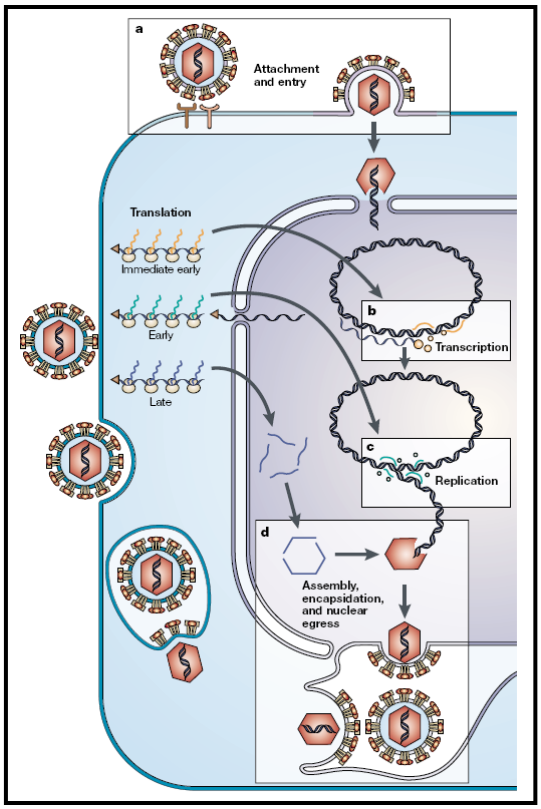


Fig.3. Schematic representation of the HSV-1 replication cycle and viral gene kinetics during lytic infection. Parental viral DNA enters the host cell nucleus and rapidly circularizes. The first genes (2-4 hours) to be expressed are the immediate–early (IE) genes (ICP0, ICP4. ICP22, ICP27, ICP47), the transcription of which, by host RNA polymerase II, is stimulated by the viral tegument protein VP16. IE proteins are transported into the nucleus and transactivate early (E) gene expression. The products of E genes include proteins that are required for viral DNA replication. DNA replication stimulates the expression of the late (L) genes, many of which encode viral structural proteins. Viral capsid assembly and progeny DNA encapsidation take place in the nucleus. Virions egress from the nucleus and the cell.

Picture courtesy of D.M.Coen and Priscilla A.Schaffer.

1.1.3.2 Viral gene transcription during latent HSV-1 infection

As HSV spreads from the primary site of infection, the virus also infects sensory neurons by fusion with the neuronal membrane at the axonal termini, and the nucleocapsid is carried by retrograde axonal transport to the nucleus in the cell body of the neuron in a ganglion. Viral DNA is released into the nucleus, in a similar mechanism to that used in lytic infection, where it circularizes. HSV DNA persists in the nucleus in a circular episomal form that is associated with nucleosomes. Lytic gene expression is repressed, but the latency-associated transcript (LAT) is expressed at high abundance, which helps to silence lytic gene expression. Latent infection was classically defined as an absence of infectious virus in ganglionic tissue accompanied by the appearance of infectious virus upon co-cultivation of the ganglionic tissue with susceptible cells (2).

LAT expression does not encode for a protein. In a recent study conducted by Umbach et al (12), it was shown that the LAT gene functions as a primary microRNA (miRNA) precursor that encodes four distinct miRNAs in HSV-1 infected cells. One of these miRNAs, miR-H2-3p, is transcribed in an antisense orientation to ICP0- a viral encoded IE protein important for productive HSV-1 replication and thought to have a role in reactivation from latency. In this study the authors furthermore identified a fifth miRNA derived from a previously unknown transcript distinct to LAT, that is complementary to ICP4. As mentioned above ICP4 is essential in active viral replication and lytic infection in order to promote transcription of most HSV-1 genes.

Although this study provided an insight on how HSV-1 suppresses lytic gene expression during latency, the field is still quite unclear. How HSV-1 promotes LAT expression in sensory neurons (thus establishing latency), and why promotes lytic gene expression in epithelial cells (thus establishing lytic infection), it is still a mystery in the virology field.

Much research has been done lately implicating a role for HDACs and histone modifications (reviewed by David M.Knipe and Anna Cliffe, 13), but the exact mechanisms still await further clarification.

1.1.4. HSV-1 manipulation of intracellular proteins and pathways

Alike many intracellular pathogens (viruses, bacteria, parasites), HSV-1 has evolutionary evolved to employ cellular proteins and modify intracellular signalling cascades to promote its replication cycle, evade immune responses, inhibit apoptosis of latent infected cells etc. Viral DNA encodes only for a small number of proteins, not enough to achieve the above and sustain survival of the pathogen inside the host. This selective pressure let to fascinating interactions between host cell and HSV-1.

Following the completion of the human genome sequence, the development of DNA microarrays for global gene expression analysis provided us with virogenomics. This systematic approach offers the potential of a large scale analysis of host-cell gene regulation and host-pathogen interactions, giving an insight for the necessity of cellular proteins essential for viral infection (14).

Considerable work using HSV-1 infection of cultured cells to study the cellular response has been done. HSV-1 infection can modulate the apoptosis response (15) and has been found to activate NF-κB pathway via IκB kinase activation (16), the Jun N-terminal kinase/stress-activated protein kinase, and p38 mitogen activated protein kinase (MAPK) cascades (17). In addition, HSV-1 genes modulate host gene expression by posttranscriptional mechanisms, altering mRNA stability, mRNA transport, and translation (18). Using DNA microarray technology Ray et al (19) studied the expression pattern of many different cellular genes in response to HSV-1 productive infection. The authors demonstrated differences in the expression of genes related to several intracellular pathways such as TGF-β, MAPK signaling genes, JAK/STAT pathway, IFN and IL1 related pathways, IGF, apoptotic and survival pathways etc. Transcription of a series of genes implicated in the above signaling pathways was either upregulated or downregulated, highlighting an important role for these signaling cascades in the establishment of a productive HSV-1 infection.

More specifically, the expression of many genes belonging to the transforming growth factor (TGF-β) signalling pathway was decreased. HSV-1 infection resulted in decreased expression of TGF-β signalling genes, such as TGF-β3, TGF-β1, Smad2, follistatin related protein (upregulated by TGF-β1 [20]), and fibulin 5 (induced by TGF-β[21]).

Moreover, genes such as TSC-22, p8 mRNA (a TGF-β responsive gene that enhances Smad transcriptional activity [22]), and latent TGF-β-BP2 (an extracellular matrix protein that targets TGF-β action [23]) also displayed reduced expression. HSV-1 infection also increased the expression of the TGF-β type I receptor gene at 12 hours post infection (hpi).

The above data, along with other studies (24, 25) point towards a significant role for the TGF-β signaling pathway in the replication cycle of HSV-1 and the establishment of productive infection.

1.1.4.1 The TGF-β signaling pathway (brief introduction)

Transforming Growth Factor-β belongs to a family of secretory polypeptides that exert a significant role in various aspects of the cell's homeostasis. This family contains various proteins structurally related, able to regulate functions such as cell migration, adhesion, differentiation, proliferation, immune response modulation and apoptosis. These responses are mostly mediated by changes in the expression pattern of key target genes. Much work has been done regarding the TGF-β signaling and its association with cancer (26).

TGFβ signaling begins by bringing together two pairs of receptor serine/threonine kinases known as the type I and type II receptors. After TGF-β binding, type II receptors phosphorylate and activate type I receptors which then transmit the signal by phosphorylating Smad transcription factors. Once activated the receptor substrate Smads transport to the nucleus and form a complex with Smad4, a binding partner common for all RSmads (27). Smad proteins possess DNA-binding activity, but the Smad4-RSmad complexes must associate with additional DNA-binding cofactors in order to achieve binding with high affinity and selectivity to specific target genes. These Smad partners are drawn from various families of transcription factors. Each Smad4-RSmad-cofactor combination targets a particular set of genes, which is determined by the presence of cognate binding sequence element combinations in the regulatory regions of target genes (28). Activated Smad complexes additionally recruit transcriptional coactivators, corepressors, and chromatin remodeling factors. Through this combinatorial interaction with different transcription factors, a common TGFβ stimulus can activate or repress hundreds of target genes at once.

2. OBJECTIVES

As mentioned above, DNA microarray studies demonstrated that productive HSV-1 infection in cell cultures modulates the expression pattern of cellular genes known to be involved in the TGF-β signaling pathway. However, there are no reports in the literature evaluating the role of TGF-β stimulation on the replication efficacy of HSV-1 *in vitro.*

In this study, we are going to exam the effect of TGF-β stimulation on the replication cycle of HSV-1 in vitro using BHK (Baby Hamster Kidney) cells and plaque assays to titrate viral particles in different concentrations of TGF-β treatment.

Specifically we are going to perform the following;

- Preparation of viral stock of HSV-1 17+ (laboratory wild-type strain)
- Titration of the prepared viral stock with plaque assay.
- Infection of BHK cells with HSV-1 17+ in the presence of 0, 1ng/ml, 5ng/ml and 10 ng/ml of TGF-β, at m.o.i 0.5 and 2.5 (multiplicity of infection) for 24hrs.
- Collection of the supernatants and titration of the viral progeny with plaque assays.
- Infection of BHK cells with HSV-1 17+ in the presence of 0, 1ng/ml, 5ng/ml and 10 ng/ml of TGF-β, at m.o.i 2.5 and 10. Immunofluorescence using antibodies against ICP0 and glycoprotein G (gG) viral proteins.
- Preparation of viral stock of HSV-1 vECFP-ICP4 (HSV-1 expressing cyan fluorescent ICP4 protein).
- Titration of vECFP-ICP4 viral stock.
- Infection of BHK cells with vECFP-ICP4 in the presence of 0, 1ng/ml, 5ng/ml and 10 ng/ml of TGF-β, at m.o.i 0.5 and 2.5 for 24hrs.
- Collection of the supernatants and titration of the viral progeny with plaque assays.

3. MATERIALS AND METHODS

3.1 Cell lines and cell cultures

BHK (Baby Hamster Kidney) cells were used. Cells were cultured in BHK medium (BHK complete medium; 500ml BHK medium, 50ml new born calf serum, 50ml Tryptose phosphate broth and 1ml penicillin/streptomycin) and incubated at 5%CO_2, 37C^0 and 80% humidity. Experiments were performed in class 2 cabinet sterile conditions to avoid bacterial and fungal infections.
BHK medium purchased from Gimco. Cells were cultured in 25cm^2, 75cm^2 and 175cm^2 cell culture flasks from Nunc. Tryptose phosphate broth (TPB) from Sigma. Penicillin/streptomycin 500x from Roche. New Born Calf Serum (NBCS by BIOSERA, UK).

3.1.1 Trypsinization

Old medium was removed from the culture flask. Cells were gently washed twice with PBS solution to achieve complete medium removal which inhibits trypsin function. For a 75cm^2 flask, 500µl of trypsin/EDTA was added. The flask was incubated at 5% CO_2 and 37^oC for approximately 3-4 minutes. Following this, the flask was microscopically observed to ensure complete detachment of the cells. The cells were then collected in 4 ml of BHK complete medium and transferred to a new flask. Stock cells were kept in -80^oC in cryotube vials provided by Nunc. (For storage 700µl cells, 200µl fetal bovine serum, 100µl DMSO by Sigma).

3.2 Viral stock preparation for HSV-1 17+ and vECFP-ICP4

A BHK confluent 25cm^2 flask was infected with 20µl of HSV-1 17+. The cells were then incubated at 37^oC and 5% CO_2 until full cytopathic effect was noticeable (48-72hrs). Detached cells and medium were then collected to a 15ml falcon tube and stored at -

80°C. Before storage, 100µl were used to infect a 175cm^2 BHK confluent flask. Again the infected cells were incubated until full cytopathic effect was observed. Detached cells and medium were again collected and stored at -80°C. Supernatants collected from the previous steps were then mixed in a 50ml falcon tube and centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatant was collected in 50ml falcon tube whereas the pellet was suspended in 1ml BHK complete medium. Supernatant was then centrifuged at 13000 rpm for 2 hours at 4°C. The pellet virions were then resuspended in 1ml fresh BHK complete medium and stored at -80°C for future use. HSV-1 isolation from the centrifuged supernatant yields CRV (Cell released viruses). The resuspended cell pellet from the first centrifuge was sonicated to release CAV (Cell associated viruses). The sonicated suspension was then filtered in sterile conditions to withhold cell debris and possible bacterial and fungal contaminants. Again CAV viral stock was stored at -80°C for future use. (Same protocol was performed for the isolation of vECFP-ICP4 HSV-1) (Fig.3.2). All above procedures were performed in class II cabinet sterile environment. (For detailed characterization of the v ECFP-ICP4 viral construct see references 29, 30). Cells were sonicated for 3 minutes. Sonicated suspension was filtered using Acradisc Syringe filters 25mm (Life sciences, UK).

3.3 Viral titration with plaque assay.

The plaque assay remains to be the most widely used technique to determine viral titres. The basis of the technique is to measure the ability of a single infectious virus to form a "plaque" on a confluent monolayer culture of cells. A plaque is formed as a result of infection of one cell by a single virus particle followed by the replication of that virus, and eventually, the death of the cell known as cytopathic effect (CPE). The newly replicated virus particles will then infect and spread to kill surrounding cells resulting in their lysis.

Viral titration was performed with plaque assay, a method that calculates the viral cytopathic effect in vitro using a series of different dilutions of the viral stock. BHK cells were cultured in 6 well plates until 100% confluence (4 x 10^5 cells). The medium was

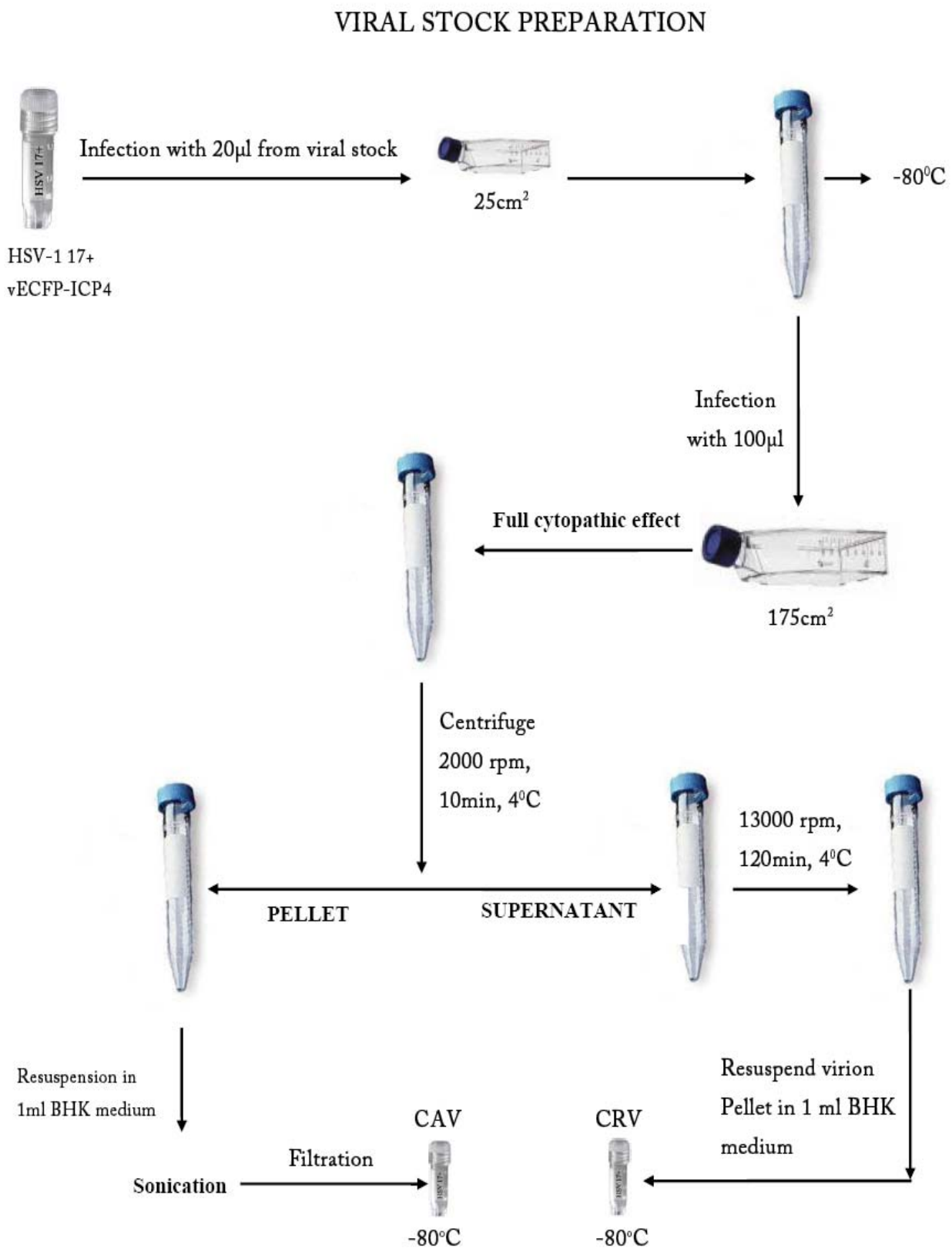
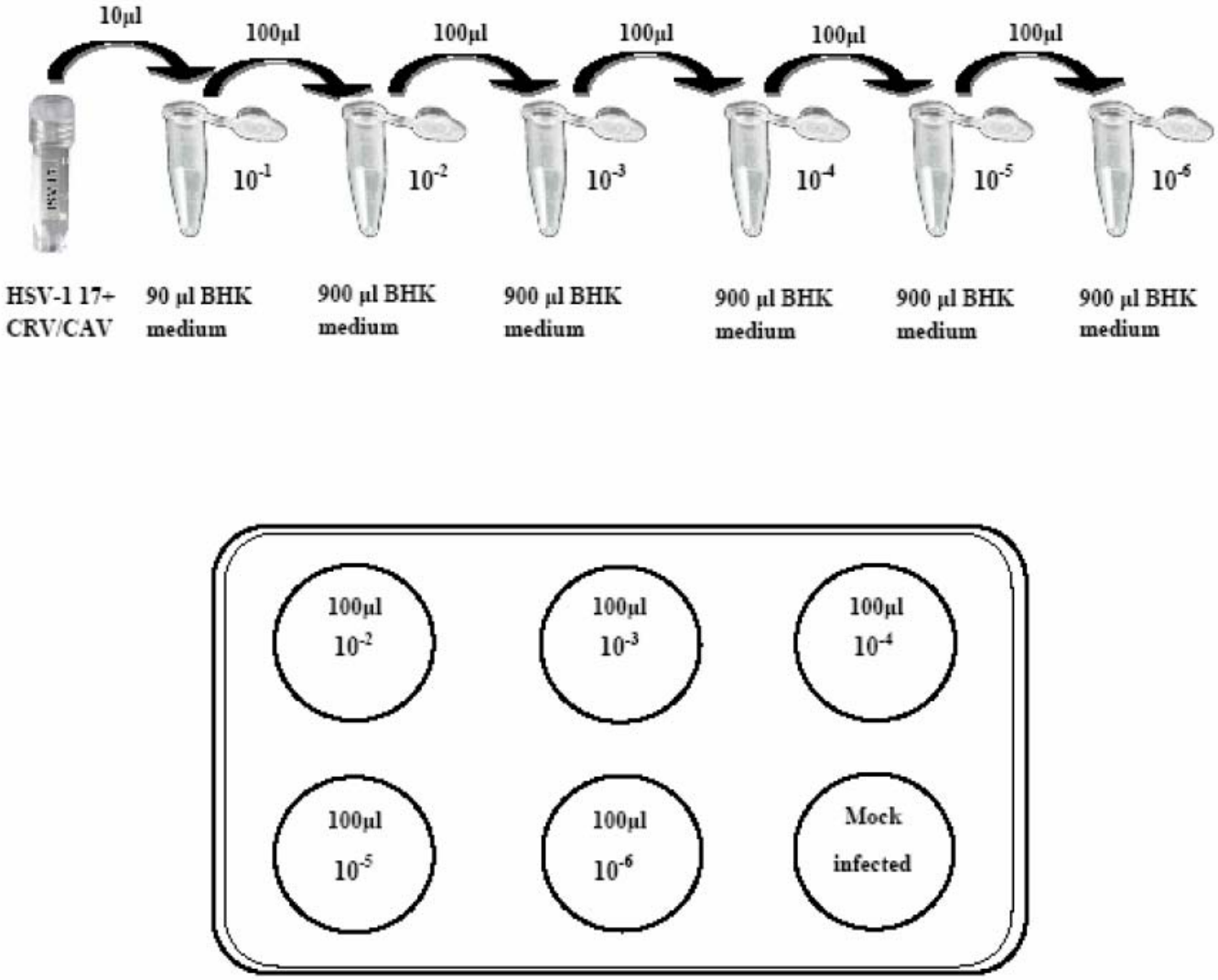


Fig.3.2. Schematic representation of the viral stock preparation protocol



Schematic representation of the method used for quantitive viral titration. A series of dilutions is performed from the initial viral stock. The dilutions are then used to infect a monolayer of BHK confluent cells in 6 wells plate. The infected cells are incubated for 48 hours and then stained with Giemsa. Plaque formations are counted with a stereoscope.

Fig.3.3 Schematic representation of plaque assay

then aspirated leaving approximately 100µl for maintaining the cell monolayer wet. Viral stocks prepared from the previous steps were thawed in ice. 10µl from each stock (CAV and CRV) were diluted in a series of dilutions from 10^{-1} to 10^{-6}. (Fig.3.3.). 100µl from 10^{-2} – 10^{-6} dilutions were used to infect five wells. The last well was mock infected with 100µl PBS.

The plate was incubated for 2 hours at 37°C, 5%CO_2 and gently moved with circular movements every 15 minutes to achieve sufficient viral cell entry throughout the whole monolayer. Approximately 10 minutes before the incubation period, the dilution of anti-HSV-1 serum was prepared (1:100). At the end of the incubation period the infective inoculum was completely aspirated and replaced by 3ml of BHK fresh medium containing 30µl of anti HSV-1 IgG serum for each well. The plate was incubated for 48hours and stained with Giemsa (approximately 500µl for each well). The cells were stained for 1 hour and gently washed with water to avoid cell detachment. The plate was left to air dry and plaques were counted using a stereoscope. Viral titre was then calculated using the plate with the most well defined plaques and counting the corresponding number of viral plaques.

3.4. Immunofluorescence

BHK cells were plated on 8 coverslips placed in a 24 well plate with 80% confluence. 1 hour prior to infection, 4 wells were treated with 5ng/ml TGF-β whereas the other 4 were treated with PBS (control). The cells were incubated for one hour and then infected with HSV-1 17+ CRV. 4 wells were infected at m.o.i 2.5 whereas the remaining 4 at m.o.i 10. The cells were again incubated at 37°C, 5% CO_2. At 2 hours post infection, 4 coverslips were removed for immunofluorescence assay with ICP0 antibodies (an HSV-1 IE protein expressed at 2 hrs after the initiation of the infection). 2 coverslips from the m.o.i 2.5 infection and two from m.o.i 10 infection (treated and untreated with TGF-β respectively). The cells were washed gently 2-3 times with PBS solution, followed by the addition of 150µl fixation solution for 10 minutes. Again the cells were carefully washed with PBS to remove remaining fixation buffer, and then treated with permeabilization

solution for another 10 minutes. Following this the coverslips were smoothly washed with PBS+1% FBS 2-3 times. Each coverslip was then incubated with 20µl of anti-ICP0 mouse antibody (antibody diluted at a concentration of 1:100) for 1 hour in dark humid environment. At the end of the incubating period the cells were washed with PBS+1%FBS to remove non binding anti-ICP0, following treatment with anti-mouse antibody Alexa 488 (diluted 1:500). Again cells were kept in humid dark environment for 1 hour and then washed with PBS+FBS1%. Coverslips were then placed in 24 well plates with approximately 150µl of PBS/FBS solution and stained with 2µl of DAPI for 5 minutes. Cells were then washed and left to air dry and then placed on a microscope slide with a drop of mountin solution. Coverslips were then fixed on the microscope slide and kept at 4°C. Fluorescence activity was tested at UV light with LEICA inverted microscope with cyan filter.

Same procedure described above was performed at 8 hours post infection for the remaining coverslips which were stained for anti-gG mouse antibody (gG is a viral structural glycoprotein of the envelope encoded at 8 hours after the initiation of productive infection).

24 well plates used for immunofluorescence assay purchased from Nunc. Anti ICP0 and anti-gG mouse antibodies from Santa Cruz, stored at 4°C. Anti mouse Alexa 488 and DAPI solution stored at -20^{0}C. Fixation, permeabilization and mountin solution were used from complete CMV pp65 antigenemia IFA kit (Light diagnostics USA). Microscope slides purchased from Menzel-Glaser and glass slides from VWR. Microscope for fluorescence detection, LEICA inverted microscope.

3.5. Infection of TGF-β treated cells and titration of progeny viruses

BHK cells were plated on 12 well plate with 100% confluence. 1 hour prior to infection with HSV-1 17+, the cells were treated either with PBS (control), 1ng/ml, 5ng/ml, or 10ng/ml TGF-β. When the incubation period with the growth factor ended, the

cells were infected with HSV-1 17+ at m.o.i 0.5 and m.o.i 2.5. The plate was incubated for 24 hours in 37°C and 5% CO_2. Then, the supernatants were collected and stored at -80°C. Supernatants were then diluted in a series a dilution and titrated for the quantification of progeny viruses in the presence or absence of TGF-β. Titration was performed by plaque assay (as described above). More specifically titration of viral progeny was performed at dilution 10^{-4}.

We used recombinant TGF-β reconstituted with sterile water and BSA

3.6. Western blot analysis of vECFP-ICP4 HSV-1 strain

Western blot analysis was used to reveal the expression pattern of vECFP – ICP4 fluorescent strain compared to its wild type counterpart (HSV 17+). Proteins tested were ICP0 and ICP4. As mentioned in discussion ICP0 is an IE protein that increases the expression of HSV genes, whereas ICP4 is an E viral encoded protein vital for the expression of E and L viral genes. HSV-1 17+ served as control.

BHK cells were infected with the viruses 17+ and vECFP-ICP4 at m.o.i 10, and whole cell extracts were prepared 3 or 6 h after virus adsorption (time points as indicated by the expression pattern of ICP0 and ICP4). The proteins were separated on 7.5% polyacrylamide gels, transferred to a nitrocellulose filter, and then detected by probing the same filter for ICP4 and ICP0 sequentially.

Whole-cell extracts of infected cells were prepared by washing cell monolayers with phosphate-buffered saline (PBS) and adding sodium dodecyl sulfate gel boiling mix. After the cell lysates were boiled, the proteins were separated by electrophoresis on sodium dodecyl sulfate–7.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose filters. The filters were blocked overnight in PBS containing 0.1% Tween 20 and 5% dried milk and then incubated with primary antibodies for 2 h in the same buffer. After being extensively washed, the filters were incubated with horseradish peroxidase- conjugated secondary antibody in PBS–0.1% Tween 20–2% dried milk for 1 h and then washed again extensively before detection of bound antibody by enhanced chemiluminescence (NEN) and exposure to film. ICP0 was detected using monoclonal antibody 11060, and ICP4 was detected by monoclonal antibody 10176.

3.7. MATERIALS

Materials used:

- BHK medium: Gibco
- Tryptose Phosphate Broth (TPB): Sigma
- New Born Calf Serum (NBCS): BIOSERA, UK
- Penicillin/Streptomycin: Roche
- Trypsin: Gibco
- DMSO: Sigma
- Fetal Bovine Serum (FBS): Gibco
- Flasks, plates, cryovials: Nunc
- Fixation, permeabilization, mountin solution: Complete CMV pp65 Antigenemia IFA Kit: Light Diagnostics USA
- Anti-ICP0 and anti-gG mouse antibodies: Santa Cruz
- Tris base: BDH
- TEMED: Sigma
- SDS: BDH
- Tween: Sigma
- Methanol: Sigma
- Nitrocellulose: 3M

4. RESULTS

4.1 Titration of viral stocks for 17+ and vECFP-ICP4 HSV-1 strains

In order to evaluate the role of TGF-β on the replicative cycle of HSV-1 we started by preparing a viral stock for both fluorescent and wild-type HSV-1 strains. Following viral stock preparation protocol we isolated the 2 strains. Following this, we next calculated the titer of the isolated HSV-1 viruses. BHK monolayer cells were plated in 6 well plates with 100% confluence. Cells were infected with 100µl 10^{-5} HSV-1,100µl 10^{-4} vECFP-1CP4 or mock infected with PBS (see materials and methods). Plaque formations were counted using stereoscope and Giemsa staining for HSV-1 17+, and inverted microscope for fluorescent counterpart. For 17+ HSV-1 strain we counted 34 plaques whereas for vECFP-ICP4, 5 fluorescent plaques were present (**Fig.4.1)**.

- **HSV-1 17+:** 34 (plaques) x 10^5 (serial dilution used) x 10 (Conversion of 100µl to ml): **3.4 x 10^7 pfu/ ml** (plaque forming units per ml).
- **HSV-1 vECFP-ICP4:** 5 x 10^4 x 10: **5 x 10^5 pfu/ml.**

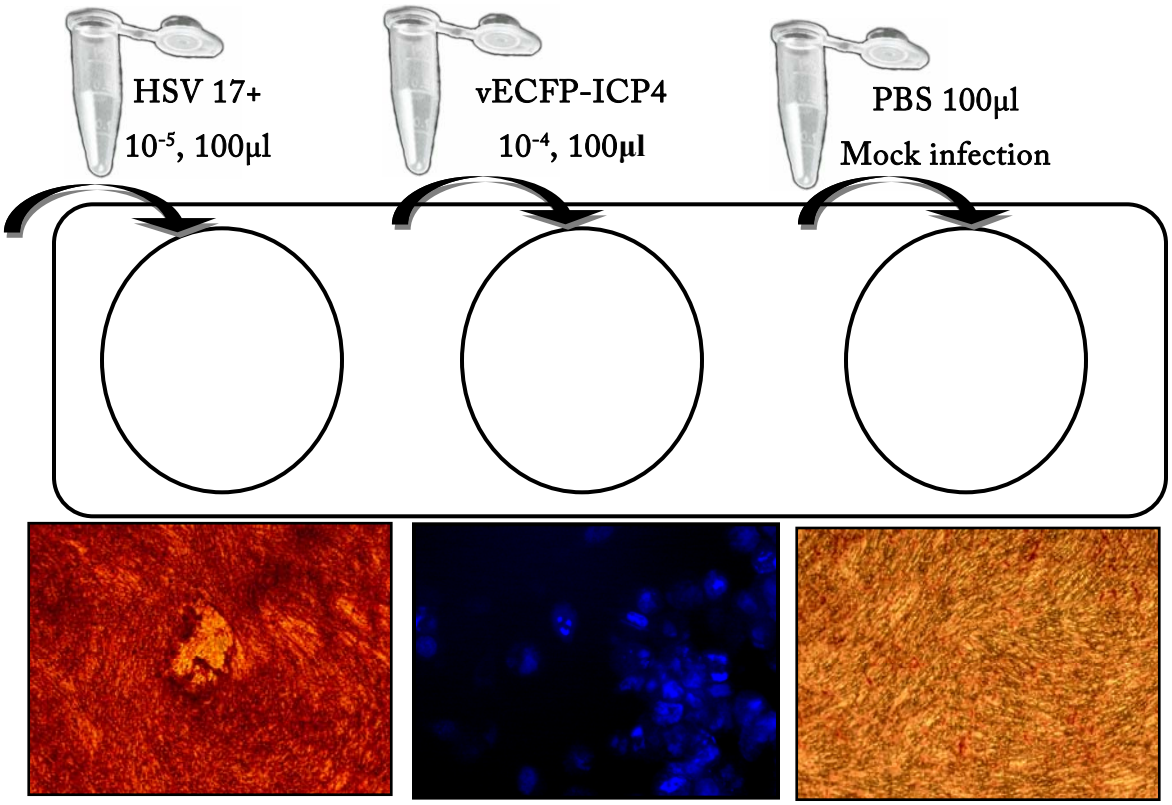


Fig.4.1. TIitration of wild type and fluorescent HSV-1 strains.**4.1 (A)** A single plaque formation from HSV-1 17+ wild type strain as shown in stereoscope after Giemsa staining. **4.1(B)** Cells infected with vECFP-ICP4 strain visualised using inverted microscope. **4.1(C)** Morphology of BHK cells mock infected with PBS after Giemsa staining.

4.2. ICP0 and ICP4 kinetics of vECFP-ICP4 HSV-1 strain

Western blot analysis was used to reveal the expression pattern of vECFP – ICP4 fluorescent strain compared to its wild type counterpart (HSV 17+). Proteins tested were ICP0 and ICP4. As mentioned in discussion ICP0 is an IE protein that increases the expression of HSV genes, whereas ICP4 is an E viral encoded protein vital for the expression of E and L viral genes. HSV-1 17+ served as control.

BHK cells were infected with the viruses 17+ and vECFP-ICP4 at m.o.i 10, and whole cell extracts were prepared 3 or 6 h after infection (time points as indicated by the expression pattern of ICP0 and ICP4). The proteins were separated on 7.5% polyacrylamide gels, transferred to a nitrocellulose filter, and then detected by probing the same filter for ICP4 and ICP0 sequentially (For details see section 3.6 Materials and methods). vECFP-ICP4 expressed normally ICP0 protein compared to 17+ wild-type strain. Alike the expression pattern of ICP4 was comparable with the wild-type strain. Differences in gel mobility regarding ICP4 between the two strains are expected due to differences in the molecular mass. These data demonstrate that fluorescent virus vECFP-ICP4 is capable of expressing replication essential proteins ICP0 and ICP4 alike its wild-type counterpart (Fig.4.2).

Fig4.2. Western blot analysis for the expression pattern of vEVFP ICP4 strain for viral proteins ICP0 and ICP4. Compared to the wild type strain, the expression of ICP0 was similar for the fluorescent counterpart. Likewise the expression of ICP4 was also normal as shown by western blot. These data pointa out that the fluorescent strain shows no replicative defects regarding the expression of these essential proteins.

4.3.1. TGF-β partially abrogated the release of HSV-1 progeny viruses

Following the titration of the prepared viral stocks, we next examined the effect of TGF-β treatment on the release of progeny viruses. BHK cells were plated on 12 well plate with 100% confluence. 1 hour prior to infection with HSV-1 17+, the cells were treated either with PBS (control), 1ng/ml, 5ng/ml, or 10ng/ml TGF-β. When the incubation period with the growth factor ended, the cells were infected with HSV-1 17+ at m.o.i 0.5. Supernatants were then diluted in a series a dilutions and titrated for the quantification of progeny viruses in the presence or absence of TGF-β. Titration was performed by plaque assay (as described above).The experiments were performed in triplicate (Fig.4.3.1.1). TGF-β treatment abrogated partially the release of HSV-1 progeny viruses for all concentrations used and for low (0.5) m.o.i. More specifically, at m.o.i 0.5, treatment with 1ng/ml TGF-β reduced viral production by 32%, 5ng/ml by 46%, and finally stimulation with 10ng/ml inhibited viral growth by 36% compared to the PBS treated control group (Fig.4.3.1.). Maximum inhibitory effect for m.o.i 0.5 was observed for cells treated with 5ng/ml TGF-β whereas complete inhibition of viral replication was not present for none of the concentrations used.

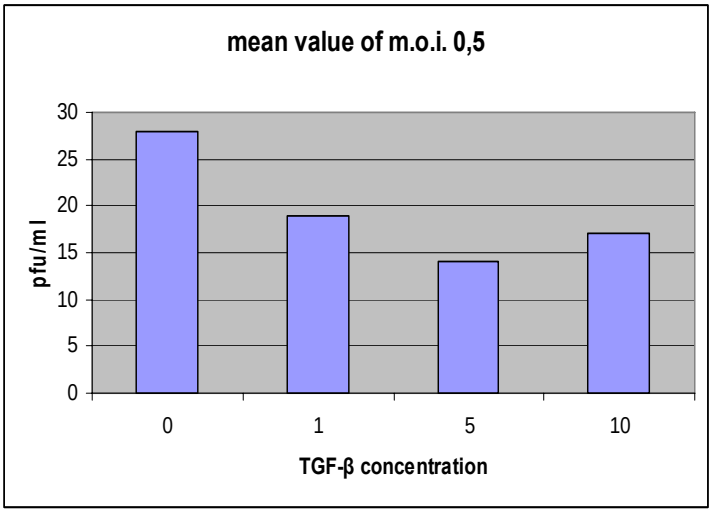
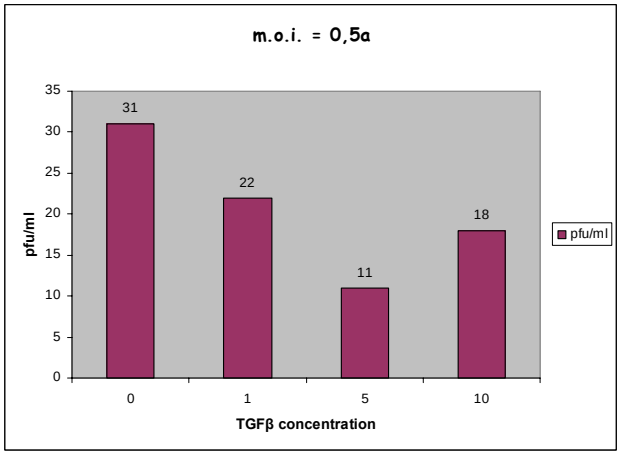
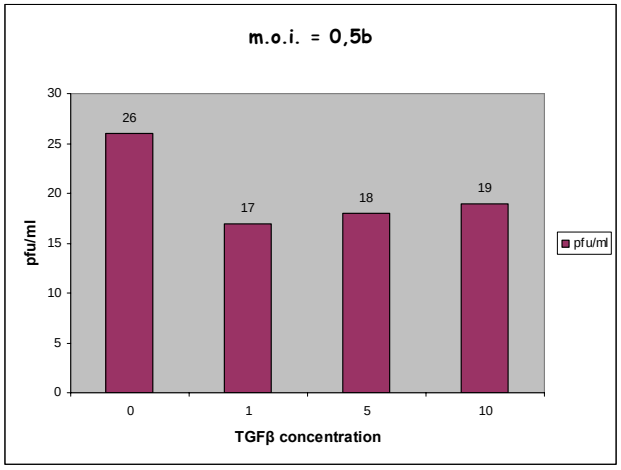


Fig.4.3.1

Fig.4.3.1. Mean values from titration assays of HSV-1 17+ progeny viruses after treatment with PBS, 1ng/ml, 5ng/ml, and 10ng/ml. For m.o.i 0.5 treatment with 1ng/ml TGF-β resulted in 32% reduction of progeny viral yield, 5ng/ml led to 46% reduction, whereas treatment with 10ng/ml inhibited viral growth by 36%. The experiments were performed in triplicate and the mean values of the number of plaques for each concentration were calculated. X-axis represents the concentrations of TGF-β. Y-axis represents the number of plaque formations.

Fig.4.3.1.1.





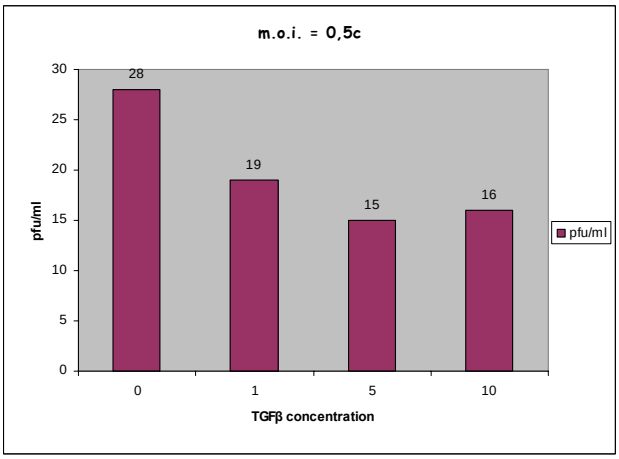


Fig.4.3.1.1. Results obtained from each one of the 3 repeats titrating viral progeny after TGF-β treatment for m.o.i 0.5. The x-axis represents the concentrations of the growth factor used to stimulate TGF-β pathway. Y-axis represents the number of plaque formations. The reduction in viral progeny yield was compared to the number of plaque formations in the PBS treated control group. For each one of the repeats the results were reproducible. TGF-β stimulation resulted in decreased viral replication with maximum inhibitory effect obtained for 5ng/ml of the growth factor.

4.3.2. TGF-β partially abrogated the release of progeny viruses at high m.o.i

We next examined the effect of TGF-β stimulation on the release of progeny viruses for cells infected with a high m.o.i 2.5. Again BHK cells were plated on 12 well plate with 100% confluence. 1 hour prior to infection with HSV-1 17+, the cells were treated either with PBS or TGF-β (same concentrations used for m.o.i 0.5). When the incubation period with the growth factor ended, the cells were infected with HSV-1 17+ at m.o.i 2.5. As for m.o.i 0.5, TGF-β treatment abrogated partially the release of HSV-1 progeny viruses for all concentrations used. Compared to the PBS treated control group, stimulation with 1ng/ml TGF-β abrogated viral yield by 25%, whereas treatment with 5ng/ml and 10ng/ml both inhibited viral replication by 43% (Fig.4.3.2). Similarly to the results obtained for m.o.i 0.5, maximum inhibitory effect was observed with 5ng/ml while none of the concentrations used resulted in complete inhibition of viral replication.

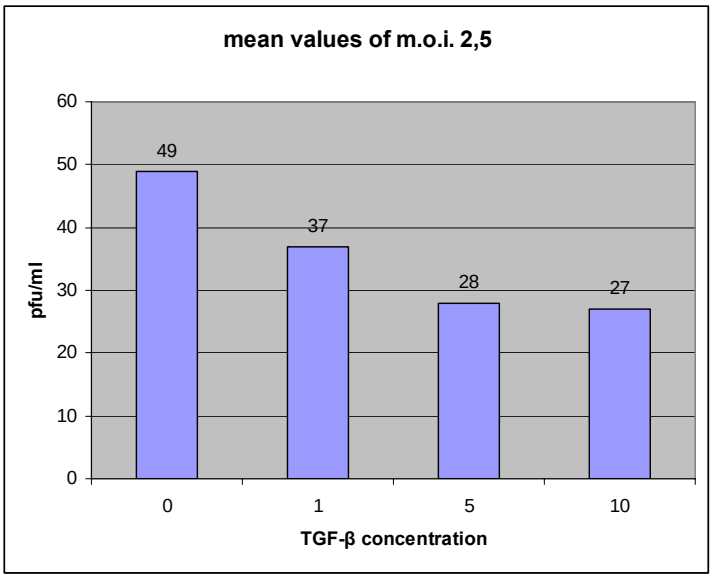
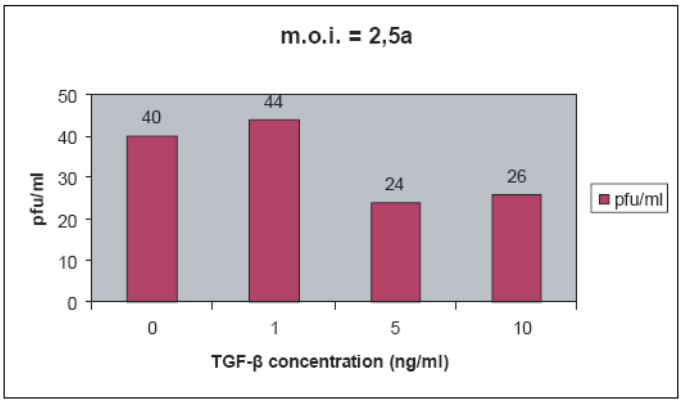
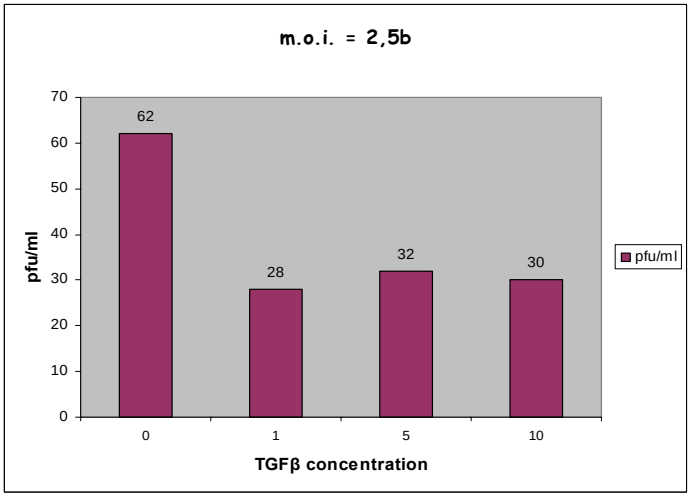


Fig.4.2.2.

Fig.4.3.2. Mean values from titration assays of HSV-1 17+ progeny viruses upon treatment with PBS (control), 1ng/ml, 5ng/ml and 10ng/ml TGF-β. For m.o.i 2.5 treatment with 1ng/ml resulted in 25% reduction in progeny viral yield whereas 5ng/ml and 10ng/ml, both inhibited viral replication by 43% compared to the PBS treated control group. The experiments were performed in triplicate and the mean values of the number of plaques for each concentration were calculated. X-axis demonstrates TGF-β concentration, Y-axis the number of plaque formations after plaque assay.

Fig.4.3.2.1





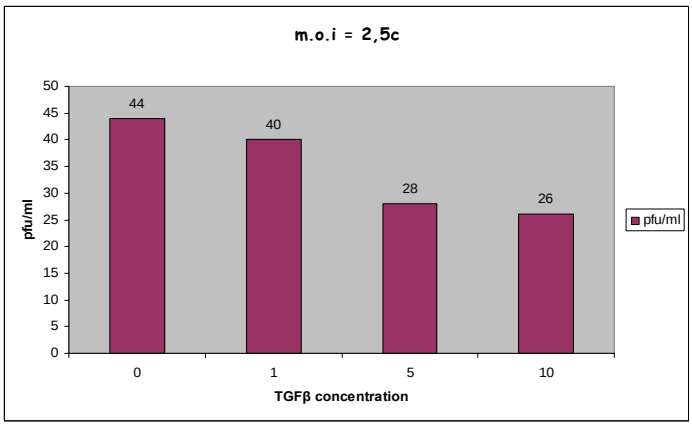


Fig.4.3.2.1. Results obtained from each one of the 3 repeats titrating viral progeny after TGF-β treatment for m.o.i 2.5. The x-axis represents the concentrations of the growth factor used to stimulate TGF-β pathway. Y-axis represents the number of plaque formations. The reduction in viral progeny yield was compared to the number of plaque formations in the PBS treated control group. For each one of the repeats the results were reproducible. TGF-β stimulation resulted in decreased viral replication with maximum inhibitory effect obtained for 5ng/ml of the growth factor.

4.4. Immunofluorescence for gG envelope protein of HSV-1 17+

To test binding activity of anti-gG antibody, BHK cells were plated on a coverslip in a 24 well plate with 80% confluence. Cells were then infected with 20µl of HSV-1 17+. The plate was incubated for 8 hours and then treated with anti-gG mouse antibody for 1 hour. Following this the cells were added anti-mouse Alexa 488 antibody for another hour, followed by DAPI staining. Coverslip was observed using inverted microscope (LEICA) (X40). (For details see materials and methods).

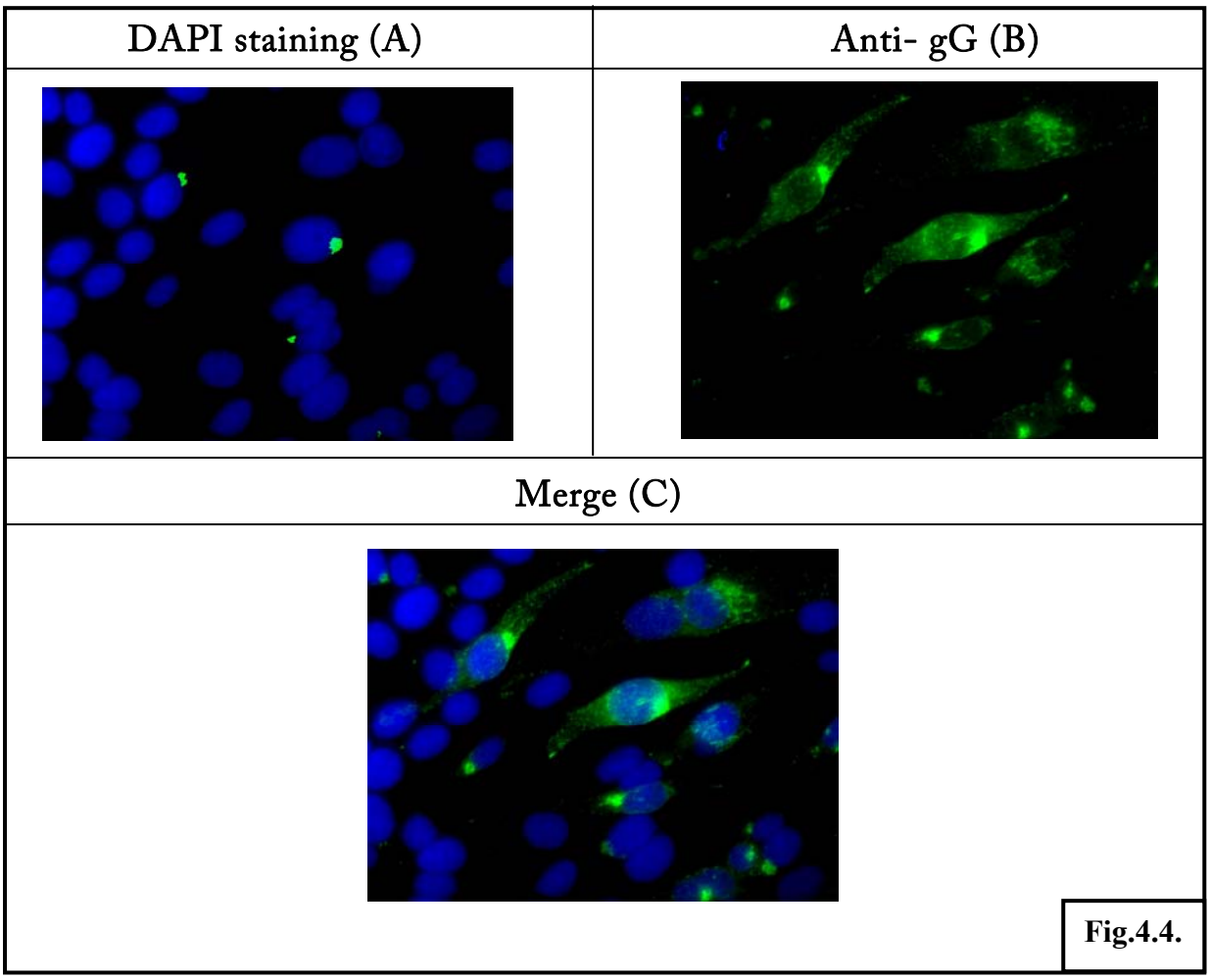


Fig.4.4. Immunofluorescence assay for gG envelope protein for HSV-1 17+. Fig 4.4 A) DAPI staining for cell nuclei. B) Staining for gG glycoprotein. With green we demonstrated the cytoplasmic localization of the gG structural protein. C) Merge picture for anti-gG and DAPI staining showing the HSV-1 infected cells.

4.4.1. Downregulation of HSV-1 ICP0 upon treatment with TGF-β

To further test the effect of TGF-β stimulation on the HSV-1 replicative cycle we performed immunofluorescence assay for the presence of ICP0 IE protein upon treatment with TGF-β or PBS (control group). BHK cells were plated on 4 coverslips placed in a 24 well plate with 80% confluence. 1 hour prior to infection, 2 wells were treated with 5μg/ml TGF-β whereas the other 2 were treated with PBS (control). The cells were incubated for one hour and then infected with HSV-1 17+ CRV. 2 wells were infected at m.o.i 2.5 (data not shown) whereas the remaining 2 at m.o.i 10. At 2 hours post infection, coverslips were removed for immunofluorescence assay with ICP0 antibodies (for more details see materials and methods).

As shown in figure 4.4.1. (B) cells pre-treated with 5ng/ml TGF-β had a decreased signal for ICP0 expression. The protein is present inside the host-cell nucleus as green dots. Treatment with PBS resulted in more abundant expression of the ICP0 protein. More cells were positive for the viral protein. Moreover, the cells presented with increased signaling comparing to the TGF-β group. Similarly to the results obtained from progeny viruses titration, TGF-β treatment resulted in partial inhibition of the viral replication but did not fully abrogated the course of infection. Same results were obtained with infection at lower m.o.i. of 2.5 (Data not shown).

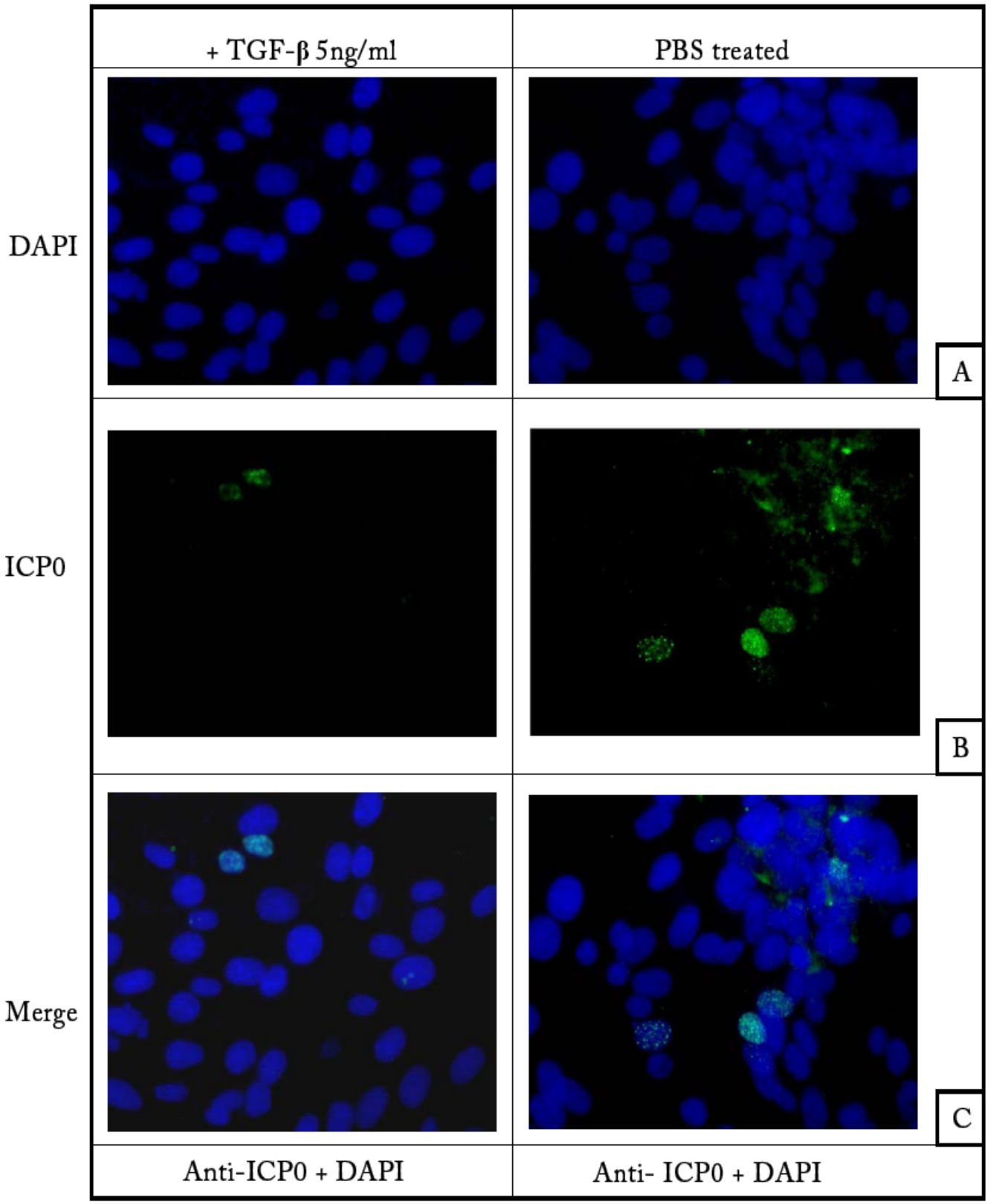


Fig.4.3.1. Immunofluorescence assay for ICP0 protein. Left column shows TGF-β pre-treated cells whereas the right column the PBS treatec control group. Row (A) shows DAPI staining of cell nuclei, (B) ICP0 staining and (C) the merged signal between the 2. TGF-β stimulation resulted in abrogated presence of the ICP0 HSV-1 protein in the infected cells. Same results were obtained for lower m.o.i 2.5 (Date not shown). Coverslips were examined using LEICA inverted microscope (x20).

4.4.2. The expression of HSV-1 glycoprotein gG is affected by TGF-β.

In order to test the effect of TGF-β stimulation on the expression of late HSV-1 genes we performed immunofluorescence assay for gG glycoprotein in cells pre-treated with TGF-β or PBS. Glycoprotein G is a late encoded structural protein of the HSV-1 envelope. BHK cells were plated on 4 coverslips placed in a 24 well plate with 80% confluence. 1 hour prior to infection, 2 wells were treated with 5μg/ml TGF-β whereas the other 2 were treated with PBS (control). The cells were incubated for one hour and then infected with HSV-1 17+ CRV. 2 wells were infected at m.o.i 2.5 (data not shown) whereas the remaining 2 at m.o.i 10. At 8 hours post infection, 2 coverslips were removed for immunofluorescence assay with gG antibodies (an HSV-1 structural protein expressed at 8 hrs after the initiation of the infection).

As shown in figure 4.4.2. (B) the results were similar to those obtained with anti-ICP0 staining. The TGF-β treated group present with decreased expression of the viral structural protein. On the other hand, the PBS treated control group showed profound expression of the viral protein with more intense signal. Again TGF-β treatment partially inhibited viral protein expression, but not fully halted HSV-1 replication. Same results were obtained for lower m.o.i 2.5 (Data not shown).

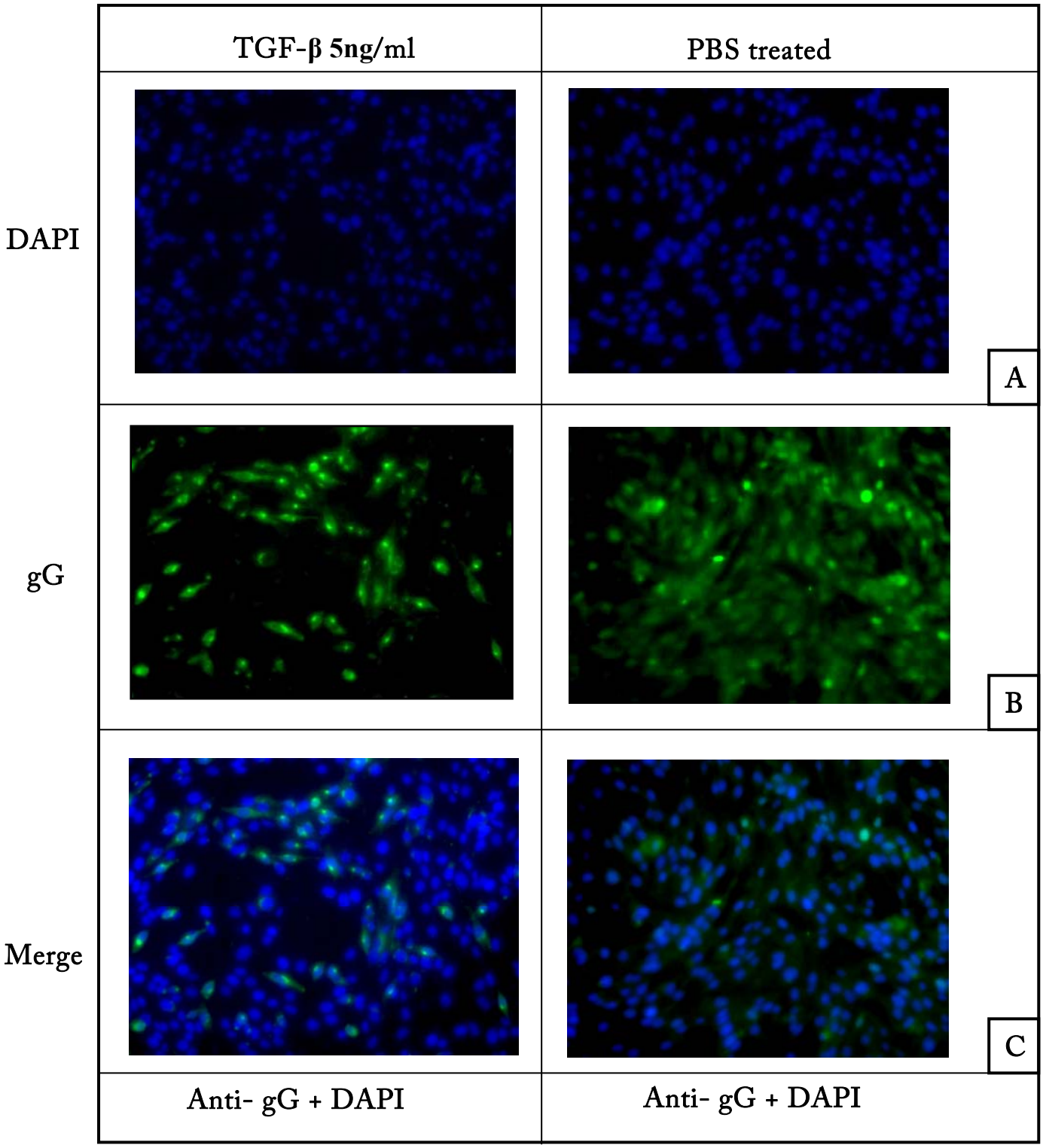


Fig.4.4.2. Immunofluorescence assay for gG glycoprotein. Left column represents the TGF-β treated group whereas the right column the PBS control group. Row (A) shows DAPI staining alone, (B) cytoplasmic localization of gG protein and (C) the merged picture between the two. As for ICP0 TGF-β stimulation abrogated the expression of the glycoprotein in infected cells, compared to the PBS treated control group. Same results were obtained for m.o.i 2.5 (not shown). Coverslips examined using LEICA inverted microscope (x20)

5. DISCUSSION

We tested the effect of TGF-β growth factor stimulation on HSV-1 progeny viral yield during lytic infection. Although recent publications indicate that HSV-1 modulates the TGF-β pathway in the course of a productive infection, there are no reports so far examining the effect of TGF-β stimulation on the release of progeny viruses.

In this study we used cell cultures permissive to HSV-1 infection, to test whether stimulation of the TGF-β pathway had an effect on the progeny viral yield. Using plaque assays as a method of viral titration, we found that cells treated with various TGF-β concentrations resulted in decreased production of viral particles. The results were similar for both high and low multiplicity of infection. More specifically, stimulation of the pathway with 5ng/ml TGF-β inhibited viral yield by 45% (maximum inhibitory effect for the concentrations tested).

These data were furthermore confirmed using immunofluorescent assay to assess the expression of 2 viral proteins. ICP0 an immediate early protein essential for viral replication and glycoprotein G (gG) a structural protein of the viral envelope encoded by the Late viral genes. Again cells treated with 5ng/ml TGF-β showed decreased signal for the presence of both viral proteins, as tested at 2 and 8 hrs post infection respectively.

We plan on continuing the experiments using a fluorescent viral strain (vECFP-ICP4) for visualization of the plaque formations upon treatment with the growth factor.

Our results are in agreement with current literature. A series of papers proved with DNA microarray studies that upon lytic HSV-1 infection, several genes implicated in the TGF-β pathway are downregulated. It is logical therefore to assume that TGF-β stimulation abrogates HSV-1 efficient replication.

However, a lot need to be done on evaluating the exact role of this pathway on the replication cycle of herpes simplex virus. A possible mechanism by which TGF-β abrogates (at least partially) HSV-1 replication could be by depriving CREB-binding protein (CBP) from virion protein VP16. As mentioned above, during HSV-1 lytic infection, more than 80 viral genes are expressed in a cascade pattern. IE gene

products are expressed from 2–4 hours post-infection. The genes that encode IE proteins are transcribed in the absence of *de novo* viral gene expression. IE gene promoters have several binding sites for cellular transcription factors, and their transcription is also activated by the virion protein VP16. This protein forms a complex with host cell factor (HCF) and localizes in the cell nucleus, where the VP16–HCF complex binds to the host transcription factor octamer-binding protein 1 (OCT1). OCT1 binds to specific sites in the upstream regulatory sequences of IE genes, tethering the VP16–HCF complex to IE gene promoters and enabling the activator domain of VP16 to recruit transcription factors that stimulate IE gene transcription. Several studies proved that VP16 virion protein, in addition to recruiting transcription factors to IE gene promoters, recruited the chromatin-modifying co-activators CBP (cAMP response element binding (CREB)-binding protein) and p300, as well as chromatin-remodelling complex (BRG1 and BRM) to modify IE genes chromatin status towards euchromatin thus promoting transcription (31).

Regarding the TGF-β signaling pathway, it has been shown that post-translational modifications of the Smad proteins are crucial in modulating their activity. More specifically Smad mediated transcription of TGF-β target genes is significantly attenuated by the direct interaction of the Smad complex with p300/CBP (CREB-binding protein), a co-activator with intrinsic acetyltransferase activity. These factors are important for efficient acetylation of Smad2, a post- translational modification that is essential for Smad2 in order to mediate TGF-β signaling (32-37).

In the notion of the above data, we can assume that TGF-β stimulation prior to infection with HSV-1, deprives competitively VP16 protein from p300/CBP factors that are essential to initiate transcription of viral IE genes. This may also explain the fact that treatment with the growth factor did not completely abolish viral replication. The fact immunofluorescence assays showed reduced signaling for ICP0 IE protein points out that the inhibitory effect exerted by TGF-β stimulation involves the early stages of HSV-1 replication cycle. Of course this necessitates further confirmation with western blot analysis. This could be a possible explanation for the inhibitory effect observed in plaque assays; however none can exclude the possibility that other mechanisms are also involved. TGF-β signaling modulates the expression of several cellular genes, which may also affect viral replication in various ways.

Modulation of the TGF-β pathway seems to be evolutionary conserved among several intracellular pathogens (Toxoplasma gondii, M.tuberculosis etc). This

highlights the central role for this factor in regulating a variety of different cellular functions and the necessity for these pathogens to modulate its course in order to achieve efficient virulence and replication. HSV-1 and other members of the herpesviruses (HHV-8 and EBV) are no exceptions. Despite the data demonstrating herpesviruses efficacy to alter the pathway for their own purposes, the exact mechanisms by which these alterations lead towards enhanced viral replication remain unclear.

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