Review Article
A Novel Class of Growth Factors Related to Herpesviruses

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Abstract

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In the last decade a novel class of growth factors related to the herpesvirus group was characterized which substantially differ from other virus-related growth factors which represent viral versions of known cellular growth factors. This novel class cannot be related to any known cellular products.

They have an ability to transform non-transformed cells *in vitro* and to suppress the transformed phenotype of transformed cells. The biological activities of these factors could be neutralized not only by antisera to corresponding virus, but, as shown with two of these factors, also by some monoclonal antibodies directed against viral gB glycoprotein. Furthermore, studies with some mutants in gene for gB revealed that this gene might be involved in growth factor synthesis.

Another characteristic making them different from other growth factors is their low molecular weight (< 10^3) and their component character, they consist of 2 or 3 active components. *In vivo* studies showed that they may influence embryonic or postembryonic development of some animals, e.g. mice, rats or fish.

Some unusual properties, e.g. extremely high titres of their biological activity demonstrated in cell cultures, or enhancement of this activity following temperature and urea treatment, or UV irradiation render them attractive for further studies indicating their peculiar structure, which is still obscure.

Pseudorabies virus growth factor, biological and physicochemical properties, transformed phenotype

Some poxviruses and herpesviruses have been shown to code for secretory proteins with structural similarity to cellular growth factors or similar polypeptides such as cytokines and chemokines. Vaccinia virus, myxoma virus, variola virus and fibroma virus encode a polypeptide which is structually homologous to both epidermal growth factor and alpha transforming growth factor (Stroobant et al. 1985; Porter and Archard 1987; McFadden et al. 1995; reviewed by Kontsek and Kontseková 2000), or orf virus encode a factor resembling vascular endothelial growth factor (Lyttle et al. 1994).

Some herpesviruses acquired the ability to code for homologs of cytokines. Cells infected with human herpesvirus 8 (HHV-8 or Kaposi's sarcoma-associated herpesvirus, KSHV) secrete protein similar to human interleukin 6 (IL-6) (Moore et al. 1996; Nicholas et al. 1997). BCFR1 gene product of Epstein-Barr herpesvirus has 89% amino acid identity with mature human IL-10 (Moore et al. 1990) and similarly equine herpesvirus type 2 codes for protein possessing high homology to human and murine IL-10 (Rode et al. 1993). IL-10 is also encoded by orf poxvirus (Fleming et al. 1997).

Some herpesviruses code for chemokines (McDonald et al. 1997; Dairaghi et al. 1998; Zou et al. 1999). For all these viral products mentioned above, the term virokines was coined (Kotwal 1999; Kontsek and Kontseková 2000). Finally, some gamma herpesviruses such as Herpesvirus saimiri, KSHV, or murine herpesvirus 68 produce D-type cyclin homologs (reviewed Laman et al. 2000).

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All these products represent viral versions of cellular growth factors, cytokines, or cyclins. Virus genes coding for them were captured from the host genome during evolution of viruses (Spriggs 1994). In the last decade a new class of growth factors related to herpesviruses appeared whose characteristics are considerably different from all known virokines. The aim of this paper is to summarize the present knowledge about these growth factors.

History and background

Although the direct role of some alpha herpesviruses in the formation of malignant tumours is questionable, if at all, there exists a body of evidence for their oncogenic potential *in vitro* (Rapp and Reed 1976; Hampar 1981), even though the mechanisms of transformation are still unknown (Galloway and McDougall 1981; 1990). Golais et al. (1985) described an original model of transformation. Human embryonic lung (HEL) cells infected with pseudorabies virus (PRV) at a low multiplicity of infection (0.001-0.01) and cultivated in the presence of antiviral antibodies and human leucocyte interferon yielded foci of morphologically transformed cells from which a stabile transformed cell line HPR-1 could be derived. Subsequent studies revealed that when a crude extract of HPR-1 cells was added to HEL cells, morphological signs of transformation were observed and this effect could be removed, when extract was treated with anti-PRV antibodies (Golais et al. 1988). Further studies have shown, that not only extracts from transformed HPR-1 cells but also those from PRV infected HEL cells devoid of infectious virus possessed such ability. Furthermore, when these extracts were added to transformed (e.g. HeLa) cells the repression of the transformed phenotype was observed (Golais et al. 1990). Similar results were achieved with herpes simplex virus type l (HSV-1 or herpes virus hominis type 1 – HHV-1) and HSV-2 (HHV-2), (Golais et al. 1992ab), as well as with some other herpesviruses (Gašperík et al. 1996).

Isolation and basic characteristics of herpesvirus related growth factors

Growth factors related to herpesviruses could be detected either in virus transformed cells, e.g. PRV related growth factor (PRGF), which was originally obtained from PRV transformed HPR-1 cells, or in cells infected with virus at low MOI and cultivated in conditions which are non-permissive for replication of virus. Such condition could be achieved during cultivation of infected cells at 41 °C or in the presence of DNA synthesis, e.g. phosphonoacetic acid (PAA). Cells infected with PRV or HSV-2 and kept at 41 °C for 4-5 days produced little or no virus and only small amounts of PRGF respectively HSGF-2 (growth factor related to HSV-2). The production of both PRGF and HSGF-2 was considerably enhanced when the temperature was shifted down to 37 °C. The reactivation of virus growth after temperature shift proceeded much more slowly than that of PRGF and HSGF-2 production, so that virus-free samples could be obtained. To remove trace amounts of virus, the media containing growth factors were acidified to pH 3, kept for 72 h at +5 °C, and afterwards their pH was raised again to neutrality (Golais et al. 1992a). PAA was shown to completely inhibit the synthesis of PRGF (Golais et al. 1990). Cells infected with PRV and HSV-2 were cultivated in the presence of PAA for 4-5 days, then the medium containing PAA was removed and replaced with intact medium. The production of both PRGF and HSGF-2 started 24 h following PAA removal. In both cases the production of both PRGF and HSGF-2 was considerably enhanced when human leucocyte interferon (IFN) was added after temperature shift or PAA removal, however, it was completely blocked in the presence of methylation inhibitor 5-azacytidine (Golais et al. 1992a).

Some cells are non-permissive for replication of some herpesviruses even in normal cultivation conditions, the cells of human origin represent such system for PRV (Golais

and Sabó 1976), or murine cells for HSV (Szántó et al. 1972). All non-permissive cell lines proved to be good producers of growth factors, when infected with an appropriate herpesvirus. MK-2 cells derived from monkey kidney (Hull et al. 1962) were shown to be the best producers of all tested growth factors (Gašperík et al. 1996). Virus replication in these cells is limited, only the trace amounts of infectious virus are shed into the culture medium, which can be successfully removed lowering pH as mentioned above.

The effect on normal and transformed cells *in vitro*

Growth factors associated with herpesviruses (HVGFs) such as PRGF, or HSGF-2 could be obtained from virus free medium of infected MK-2 cells (Gašperík et al. 1996). Both crude media and more or less purified samples of HVGFs (purification procedures will be mentioned later) have a transforming ability, when added to normal, non-transformed cells. The cells cultivated in the presence of HVGFs acquire the transformed phenotype ("criss-cross" pattern of growth) and an ability to form colonies in soft agar (anchorage-independent growth) see Plate III, Fig. 1ab. This effect is reversible, HVGFs removal leads always to reappearance of an original, non-transformed phenotype (Golais et al. 1990; 1992a; 1992b; Gašperík et al. 1996).

Dušinská et al. (1994) studied the transforming activity of PRGF in detail using the in vitro Syrian hamster cell transformation assay (Pienta et al. 1977). The transformation frequency ranged from 0.1 to 0.26 % without any relation do PRGF concentration.

Pieklo et al. (1999) studied the interactions of PRGF with ovarian cells in vitro. Granulosa cells isolated from porcine ovaries were cultivated as monolayers for 6 days in an intact medium and in medium supplemented with PRGF. Increased population density and change towards more fibroblast-like shape of cells was observed when PRGF was present. Furthermore the cells divided significantly faster and an increase of progesterone production was observed. The rise of progesterone content was not connected with increased number of secretory cells, but with a stimulation of production per cell.

An opposite effect, a repression of the transformed phenotype was observed in transformed cell lines cultivated in the presence of HVGFs, the cell morphology resembled that of non-transformed cells lost the ability to form colonies in soft agar (Plate IV, Fig. 2ab). This effect was similarly reversible, bound to the presence of HVGFs in the medium (Golais et al. 1990; 1992a; 1992b).

In order to understand better both phenomena, the transforming and transformed phenotype repressing activity of PRGF, Urbančíková et al. (1999) studied actin cytoskeleton and its alterations by PRGF using normal human fibroblasts VH-10 and transformed HeLa cells. The importance of the actin cytoskeleton in cellular processes giving rise to transformed phenotype was clearly demonstrated by Pollack et al. (1975). The changes in actin filament composition mediated by PRGF in VH-10 cells proceeded very fast (Plate V, Fig. 3ab), they could be detected already after 10min. In comparison to untreated cells the staining of treated cells was more diffuse and a number of actin microfilaments in individual stress fibers became reduced. These changes resembled those observed in transformed cells. An opposite process was induced in HeLa cells: the number of filamentous actin structures increased (Plate VI, Fig. 4ab). Whether the interaction of PRGF with actin structures was direct, or through the regulation proteins of the cytoskeleton assembly, or on the level of gene expression remains to be answered.

The transformation repressing activity was further studied in combination with antiproliferative activity of various cytostatics. An undesirable effect was observed, when PRGF was combined with 11 out of 12 cytostatics. These cytostatics suppressed the ability of PRGF to change the transformed phenotype, and on the other hand, PRGF diminished the effect of these drugs on growth and metabolism of the cells. Only in the case of methotrexate

both suppression of transformed phenotype and inhibition of cell growth were observed (Kocáková et al. 1997).

A possible role of gB gene

Several experimental data support the idea that a gene coding for gB glycoprotein might be involved in HVGFs synthesis. PAA and tunicamycin completely inhibited PRGF production (Golais et al. 1990). As PAA is known to inhibit the synthesis of herpesvirus structural proteins (Boetzi 1979), and tunicamycin inhibits N-glycosylation (Katz et al. 1980; Norrild and Pedersen 1982), it was assumed that glycosylation might play at least an indirect role in PRGF synthesis. Both transforming and transformed phenotype repressing activity could be removed with antiserum against corresponding virus (Golais et al. 1990).

To shed more light upon this problem a panel of monoclonal antibodies (moabs) directed against various glycoproteins of HSV-1 and HSV-2 (Bystrická et al. 1991) was tested for ability to neutralize both transforming and transformation repressing activity of HSGF-2. Two moabs, No. 170, directed against gB1,2 and No. 499, directed against gB2 neutralized both activities of HSGF-2 (Golais et al. 1992a). Similarly, the activity of PRGF could be successfully removed by two anti-gB (gII) moabs of PRV (Gašperík et al. 1994). It was the first sign supporting the idea, that gB gene might be involved in the synthesis of HVGFs. The experiments with gB gene recombinants of HSV-1 strengthened this hypothesis. The ability of HSV-1 strains to produce HSGF-1 was shown to be associated with the syn+ phenotype (cell rounding). The syn (syncytial) strains were not able to produce this factor. There are several loci spread through the genome, responsible for the syn phenotype, of HSV-1, one of hem, the syn3 locus is located within the gB gene of HSV-1 (DeLuca et al. 1982; Weise et al. 1987). A syncytial ANGpath strain is not able to produce HSGF-1, an intratypic recombinant of this strain containing syn3 locus from non-syncytial KOS strain acquired the ability of cell rounding (syn+) along with the ability to produce HSGF-l (Golais et al. 1992b), thus indicating that gB gene might be involved in the synthesis of this factor. Other experiments which will be reported later also demonstrated that glycosylation plays an important role in both biological and physicochemical properties of PRGF and HSGF.

HVGFs consist of two or three components

Purification of PRGF by discontinuous recycling chromatography (Morávek 1971) revealed that this factor consists of two active components, $PRGF_A$ and $PRGF_B$. Both of Mr < 1000.Both components used separately possessed only the transforming activity, however, for transformation repressing activity either non-resolved PRGF, or both components applied simultaneously were recquired (Gašperík et al. 1994).

Using the same purification method, HSGF-1 and HSGF-2 were shown to consist of three components, $HSGF_A$, $HSGF_B$, and $HSGF_C$. Whereas the A and C componets possessed only transforming activity, B component was devoid of this activity, it has only an ability to repress the transformed phenotype, thus, it might be an appropriate candidate for treatment of tumours *in vivo*. In addition to PRGF and two HSGFs, other seven human and animal herpesviruses were tested for HVGFs production and their component character. Six of them could be resolved into two and one into three components (Gašperík et al. 1996).

Monosaccharides and cations present in the medium of cells producing HVGFs proved to be important both for their biological properties and their component characteristics. PRGF is normally produced in PRV infected MK-2 cells fed with glucose. So prepared PRGF consists of $PRGF_A$ and $PRGF_B$ components. The replacement of glucose by other monosaccharides lead to three outcomes:

1. Production of normal, fully functional PRGF, however resistant against two anti-gB moabs 36 and 68 (Qvist et al. 1989), neutralizing the activity of PRGF produced in the presence of glucose;
2. production of PRGF with the one-component character retaining the transforming and devoid of transformation repressing activity, and
3. complete inhibition of PRGF production.

Two-component PRGF was produced e.g. in the presence of galactose, or manose, on the other hand, only one component was produced in the presence of fructose or fucose. It is interesting to note, that one-component PRGF produced in the presence of fucose could be neutralized with moab No 36, but not with moab No 68. In the presence of ribose and lyxose no PRGF was produced. Furthermore, the production of PRGF was shown to depend on the presence of Ca^{2+} ions, however, it was not influenced by Mg^{2+} ions (Kocáková et al. 1996). Similarly, galactose and fructose gave rise to two-component HSGF-2 resistant to moabs No. 170 and 499, arabinose, xylose and ribose produced one-component HSGF-2 which could be neutralized only with moab No 170 and HSGF-2 production was inhibited in the presence of fucose. Different results were obtained when HSGF-2 was produced in the presence of three cytostatics, in this case similarly only one-component factors were produced, however, as different from those produced in the presence of corresponding monosaccharides, their transforming activity could be neutralized with the moab No. 499 and not No.170 (Živicová et al. 1998). These finding support the idea mentioned above, that the process of glycosylation plays an important role in HVGFs synthesis.

Some atypical physicochemical properties of PRGF and HSGF-2 suggesting their peculiar structure were demonstrated by Golais et al. (1996) and Živicová et al. (1998). The temperature and urea treatment resulted in enhancing of the biological activity of both factors, and similar enhancing effect was observed following UV irradiation. Heparin completely destroyed the suppressing effect on the transformed phenotype, however without affecting the transforming activity. Both PRGF and HSGF-2 proved to be completely resistant to detergents and lipid solvents.

PRGF influence the postnatal development of mammals and embryonic and larval development of fish

$PRGF_A$ administered subcutaneously was shown to enhance growth and to facilitate the postembryonic development of BALB/c mice. PRGF-treated animals grew much more rapidly as compared to the mock-treated ones whose growth did not substantially differ from intact, non-treated animals. The initiation of hair growth appeared about 4 days sooner in PRGF-treated than in control mice. All $PRGF_A$ samples lost their ability to enhance the growth of mice when treated with moabs No 36, 68, or both (Qvist et al. 1989). The susceptibility of animals markedly decreased with age. Mice aged 48 hrs showed highest susceptibility, their body mass was about 100-150% higher as compared to mass of mock-treated animals. At the age of 12 days all animals became non-susceptible to $PRGF_A$.

A similar growth stimulating and development facilitating effect was observed, when $PRGF_A$ was introduced subcutaneously into 3-day-old WISTAR rats and this effect was similarly neutralized by two moabs against gB of PRV (Csabayová et al. 1995).

Kovrižnych et al. (1998) studied the effect of non-resolved PRGF on embryonic and larval development of zebrafish (*Danio rerio*) and juveniles of guppy (*Poecilia reticulata*). The exposure of zebrafish eggs to PRGF at higher concentrations (1×10^7 to 1×10^{13}) significantly slowed down the development of juveniles and retarded their growth. On the other hand, PRGF at lower concentrations (1×10^5 to 1×10^3) significantly stimulated the growth, however, as different from mice and rats in which the enhanced outgrowth was proportional without any preference to individual organs or tissues, zebra fish exposed to

PRGF exhibited deformations in the cranial part of the body. Study on guppy revealed no significant differences between PRGF-treated and non-treated fish.

Conclusions

HVGFs whose synthesis was demonstrated either in herpesvirus transformed, or in semipersistently, or abortively infected cells represent a new class of growth factors whose structure and composition are thank to their peculiar physicochemical properties rendering them unaccessible to conventional methods still to be uncovered. The ability of these factors to suppress the transformed phenotype of cells in vitro, especially the B components of the three- component-factors lacking the transforming activity might find the usage in cancer therapy. Further, the ability to enhance growth of granulosa cells their progesterone production might find application in hormone production *in vitro*. Similarly the ability to enhance the growth and facilitate the postnatal development of animals appears to be very tempting for further studies, as it might find some applications in animal husbandry. However, a great disadvantage of these factors is a failure of our present attempts to disclose their structure which appear to be very peculiar and needs some novel, unconventional approaches.

Nová trieda rastových faktorov asociovaných s herpetickými vírusmi

V poslednom desaťročí bola charakterizovaná nová skupina rastových faktorov asociovaných s herpetickými vírusmi. Tieto rastové faktory sa podstatne líšia od iných vírusových rastových faktorov, ktoré reprezentujú vírusovú verziu známych bunkových rastových faktorov. Tieto novoobjavené rastové faktory nemôžu byť spájané so žiadnym známym bunkovým produktom.

Majú schopnosť transformovať bunky in vitro a potláčať transformovaný fenotyp rakovinových buniek. Biologické aktivity týchto faktorov môžu byť neutralizované nielen antisérom k danému vírusu, ale ako sa ukazuje u dvoch z týchto faktorov, aj niektorými monoklonovými protilátkami namierenými proti vírusovému gB glykoproteínu. Okrem toho, štúdie s niektorými mutantmi v gB géne naznačujú, že tento gén by sa mohol podieľať na syntéze týchto faktorov.

Líšia sa od iných rastových faktorov molekulovou hmotnosťou ($<10^3$) a komponentovým charakterom, pretože pozostávajú z dvoch alebo troch aktívnych komponentov. Štúdie in vivo ukázali, že majú vplyv na embryonálny a postembryonálny vývoj niektorých zvierat, napr. myší, potkanov a rýb.

Niektoré neobvyklé vlastnosti ako napr. dosiahnutie extrémne vysokých titrov ich biologickej aktivity na bunkových kultúrach, alebo zvýšenie ich biologickej aktivity vplyvom teploty, močoviny alebo UV žiarenia ich robia zaujímavými pre ďalšie štúdium, ktoré smeruje k objasneniu ich štruktúry, ktorá doposiaľ zostáva neznáma.

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Plate III
Konvalina I. et. al.: A Novel ... pp. 29–36

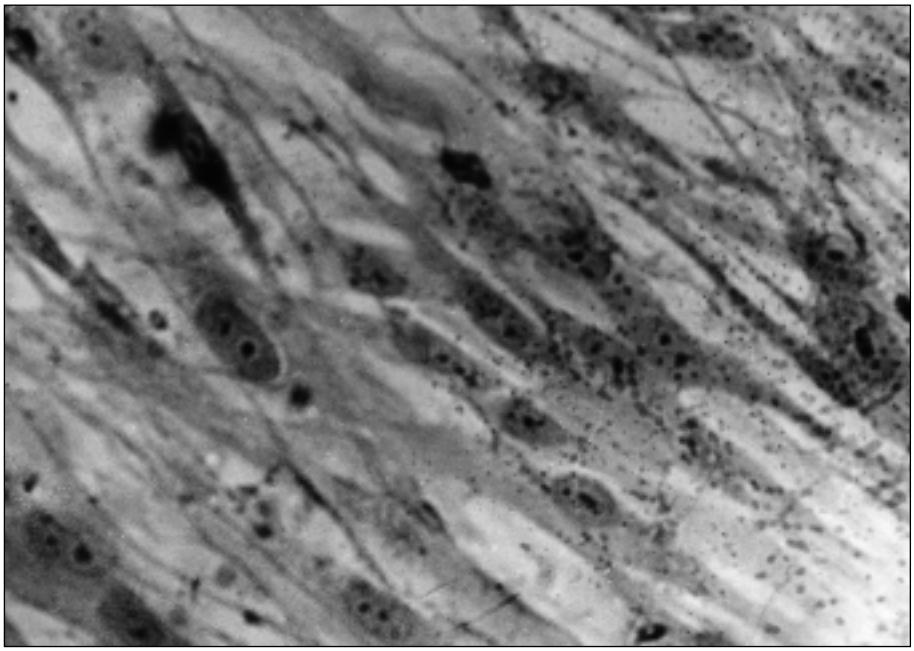

Fig. 1a. Normal human embryonic (HEL) cells. (× 400).

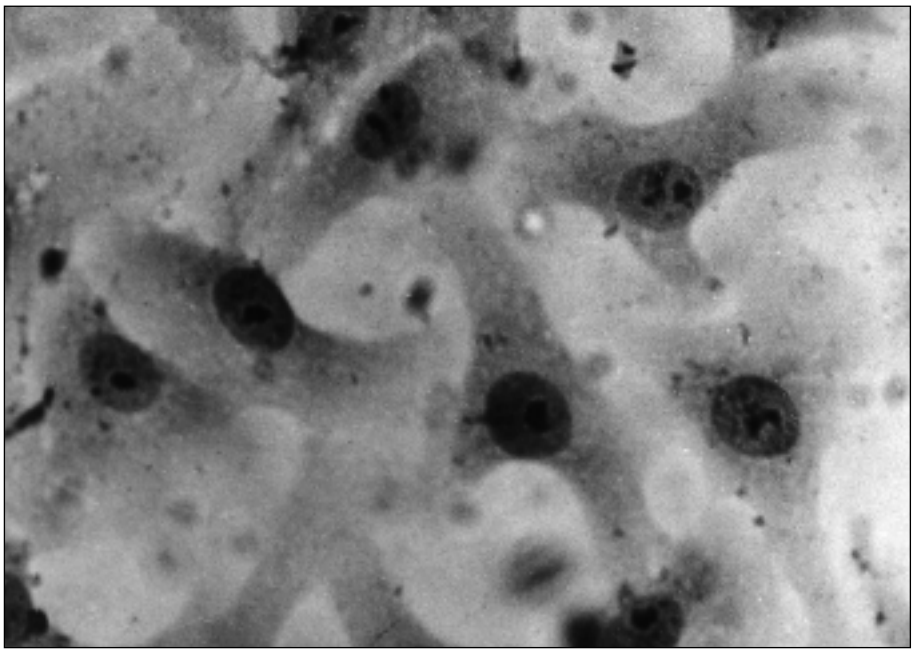

Fig. 1b. Transformed HEL cells (cultivated in presence of herpesvirus related growth factor). (× 400).

Plate IV

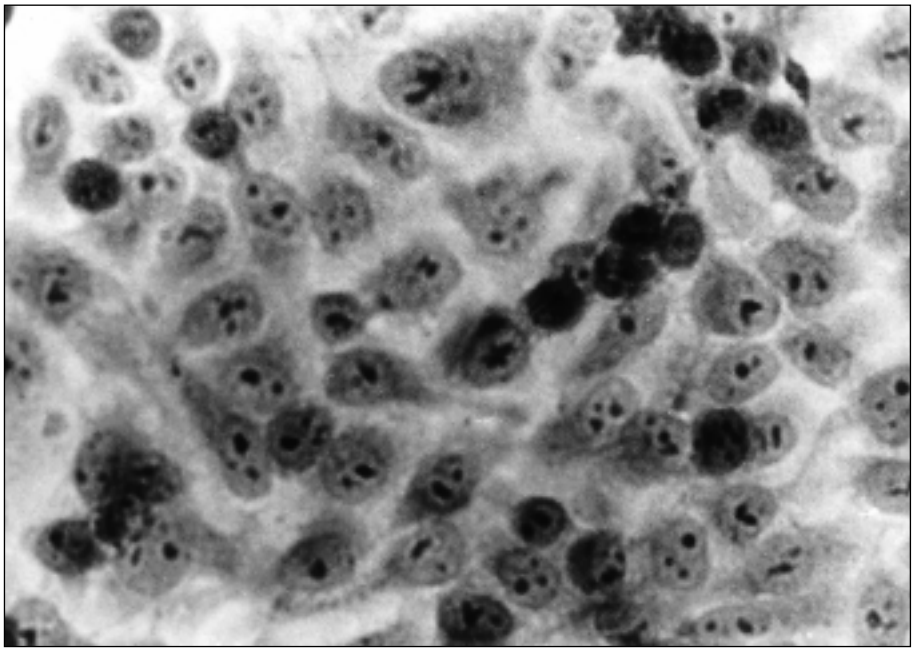

Fig. 2a. Transformed HeLa cells. (× 400).

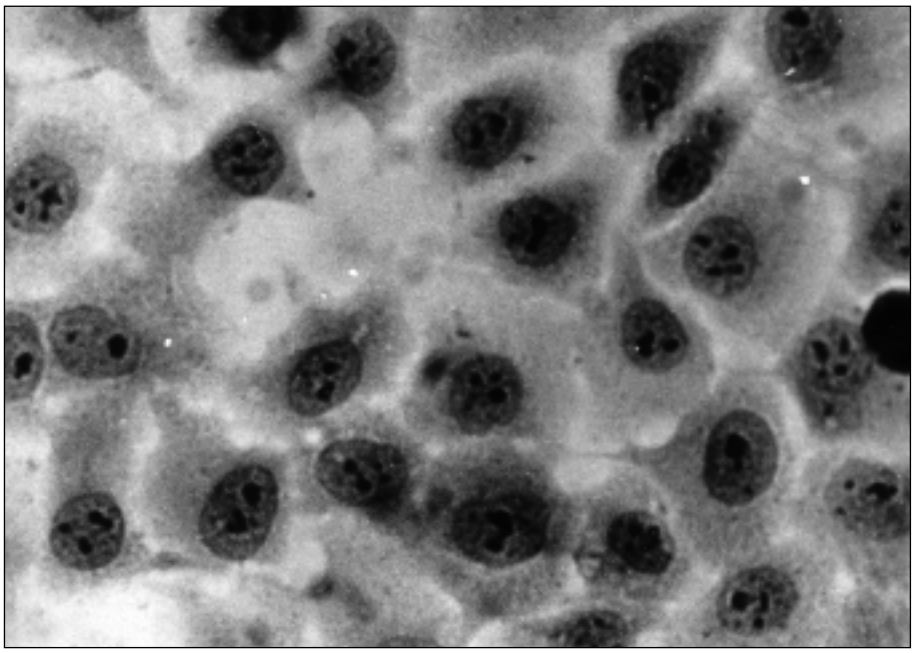

Fig. 2b. Repressed phenotype of HeLa cells (cultivated in presence of herpesvirus related growth factor). (× 400).

Plate V

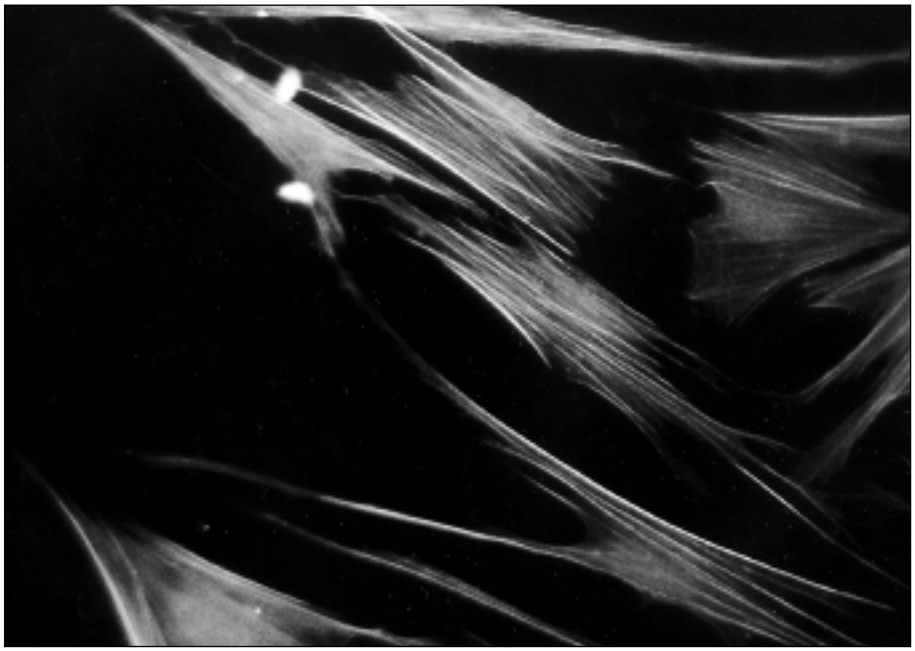

Fig. 3a. Control VH-10 cells (× 400).

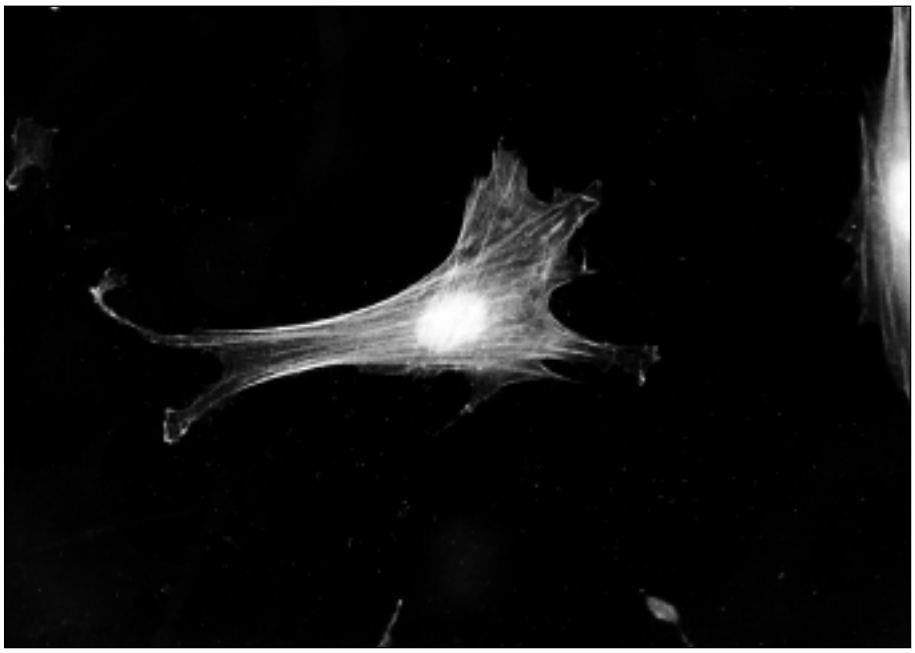

Fig. 3b. VH-10 cells cultivated 10 min in presence of unresolved PRGF (× 400).

Plate VI

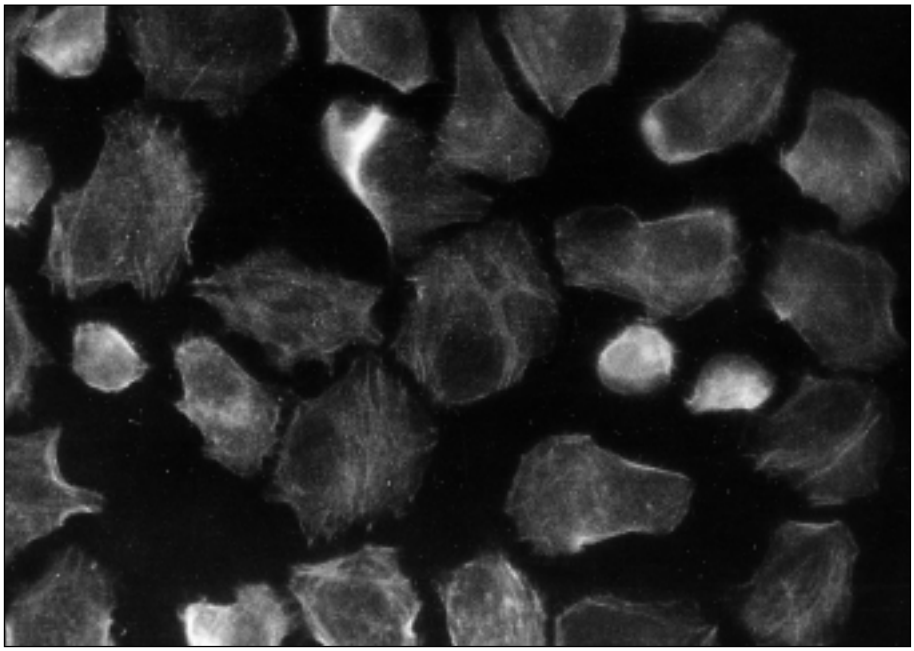

Fig. 4a. Control transformed HeLa cells (× 400)

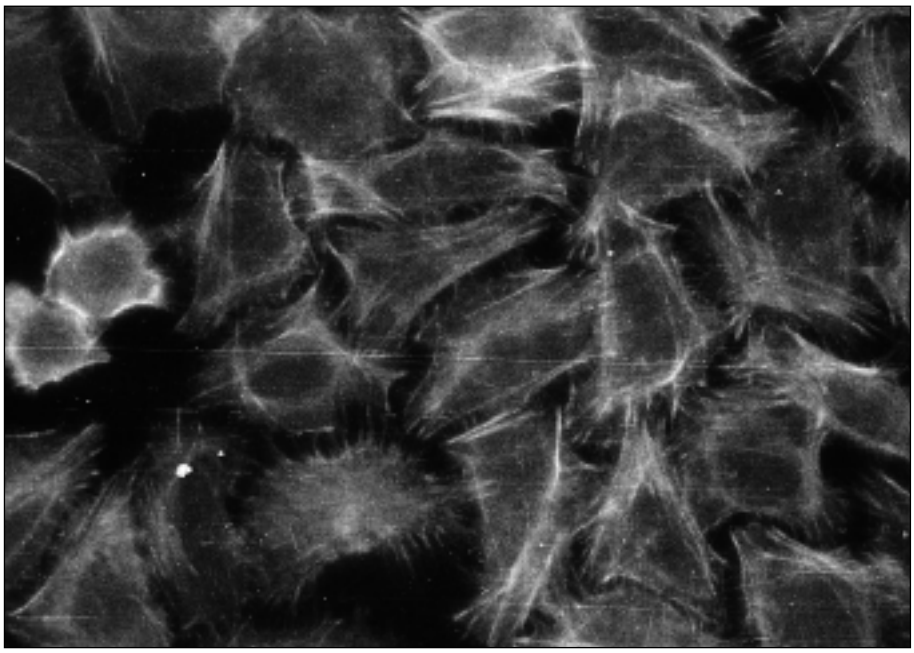

Fig. 4b. Transformed HeLa cells cultivated 24 h in presence of unresolved PRGF (× 400).