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Involvement of integrin-linked kinase in capillary/tube-like network formation of human vascular endothelial cells

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Abbreviations: ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; ILK, integrin-linked kinase; KD, kinase deficient; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; VEGF, vascular endothelial growth factor.

ABSTRACT

Angiogenesis is a complex process involving an ECM and vascular endothelial cells (EC), and is regulated by various angiogenic factors including VEGF. The ability to form a capillary/tube-like network is a specialized function of EC. Therefore, *in vitro* angiogenesis was assessed by a capillary/tube-like network formation assay. There are three angiogenic parameters: capillary length, number of capillaries, and relative capillary area per field. We evaluated capillary length per field in the assay. VEGF promoted capillary/tube-like network formation of EC in a type I collagen gel matrix *in vitro*. Moreover, we demonstrated the involvement of ILK in a VEGF signaling pathway mediating capillary/tube-like network formation of EC using dominant-negative, kinase deficient ILK. This is a straightforward assay to monitor responses of human vascular endothelial cells.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a critical factor in many diseases, such as diabetic retinopathy, rheumatoid arthritis, and tumor progression (1). On the other hand, neovascularisation during embryonic development is distinguished from angiogenesis. Angiogenesis is a complex process involving extracellular matrix (ECM) and vascular endothelial cells, and is regulated by various angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-8 (IL-8), transforming growth factor-α (TGF-α), and others (2, 3). These factors can promote cell proliferation, chemotactic migration, and capillary/tube-like network formation of vascular endothelial cells *in vitro* and *in vivo*. The endothelial-specific mitogen VEGF has been shown to be a key positive regulator of tumor angiogenesis including glioblastoma, colon, breast, renal, and other tumors (4-6). The ability to form a capillary/tube-like network is a specialized function of vascular endothelial cells. VEGF also promoted

formation of a capillary-like network of vascular endothelial cells in various matrix gels such as collagen and fibronectin *in vitro*. The response of vascular endothelial cells to soluble angiogenic factors, as well as interactions between ECM and the cells were required for new blood vessel formation.

Integrin adhesion receptors are $\alpha\beta$ heterodimeric transmembrane glycoproteins that interact with the ECM and cytoplasmic molecules including cytoskeletal and catalytic signaling molecules (7-9). The integrin-linked kinase (ILK) is a serine/threonine protein kinase that was initially identified as the integrin β1-tail-binding protein (10). The C-terminal region of ILK exhibits significant homology to other protein kinase catalytic domains and ILK can phosphorylate protein substrates such as integrin β1 cytoplasmic region, Akt/protein kinase B (PKB), and glycogen synthase kinase-3 (GSK-3) (11, 12). It has been reported that integrins cooperate with the VEGF receptors to promote activation of an *in vitro* angiogenic program in vascular endothelial cells (13). Furthermore, ILK transduces signals affecting anti-apoptosis and cell cycle progression, and these are mediated by interactions between integrins and the ECM (14, 15). However, so far no biological function of ILK in vascular endothelial cells has been demonstrated.

In this study, we investigate the effect of dominant-negative, kinase deficient ILK (ILK-KD) in the capillary/tube-like network formation of human umbilical vein endothelial cells (HUVEC). We demonstrate a critical role for ILK in network formation by vascular endothelial cells in a collagen gel matrix.

MATERIALS AND METHODS

Cell culture and reagents

Human umbilical vein endothelial cells (HUVEC) were obtained from Cell Systems (Kirkland, WA) and were cultured in collagen-coated flasks (Becton, Dickinson and Company, Franklin Lakes, NJ) in CS-C medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), according to the instructions of the supplier. Cells were passaged at 37°C in an atmosphere of 5% CO_2. The cells from passage 3-7 were used for capillary/tube-like network formation assay on type I collagen gel matrix. RPMI-1640 medium was obtained from Sigma (St Louis, MO). Recombinant human vascular endothelial growth factor (rhVEGF) was purchased from R & D Systems (Minneapolis, MN). Type I collagen gel matrix (Cellmatrix type I-A) was from Nitta Gelatin Inc. (Osaka, Japan). LY294002, a selective PI 3-kinase inhibitor, was obtained from Calbiochem (San Diego, CA).

Plasmid constructions

We used a basic procedure. Briefly, extraction of cytoplasmic RNA from HUVEC was performed using TRIzol solution (Life Technologies Inc., Grand Island, NY). The reverse transcription of RNA was performed in a reaction mixture containing total RNA, AMV reverse transcriptase (Promega, Madison, WI), oligo$(dT)_{15}$, ribonuclease inhibitor, and each dNTP mixture. The mixture was incubated at 42°C for 30 minutes, heated to 95°C for 5 minutes, and then chilled at 4°C for 5 minutes. First strand cDNA (corresponding to 1 μg of total RNA) was subjected to amplification using the forward primer 5'- GAG ACC CAA GCT TCT GGA TGG ACC TGG AAG CCT -3', the reverse primer 5'- GAT GAC GCG GCC GCT CAG GTG CCT TTG CTT TCT G -3' and high fidelity pfu DNA polymerase (Promega). After an initial cycle of denaturation at 95°C for 1 minute, polymerase chain reaction was carried out for 25 cycles with the following cycle conditions: 95°C, 30 seconds; 55°C, 30 seconds; 72°C, 3 minutes with a postincubation of 72°C for 5 minutes. The PCR product was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The insert of recombinant plasmid, pcDNA3-ILK, was verified by restriction endonuclease digestion and DNA sequence analysis using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Site-directed mutagenesis

Point mutation was generated into pcDNA3-ILK using GeneEditor in vitro Site-Directed Mutagenesis System (Promega), according to the manufacturer's instructions. Mutant oligonucleotide (with the altered nucleotide underlined) was used to change glutamic acid at position 359 to a lysine (E359K, 5' - TGG GTA GCC CCC AAA GCT CTG CAG AAG - 3') within the kinase domain (16). The sequences of the mutant plasmid (pcDNA3-ILK-KD) were confirmed by DNA sequencing. Transient transfections were performed with FuGENE6 Transfection Reagent (Roche Diagnostics, GmbH,

Germany) 18 hours prior to VEGF stimulation, according to the manufacturer's instructions.

In vitro capillary/tube-like network formation assay on type I collagen gel matrix

Endothelial capillary/tube-like network formation was assessed using type I collagen gel matrix. Eight volumes of 0.3% Cellmatrix type I-A solution (Nitta Gelatin, Inc.) were mixed with one volume each of reconstituted buffer (4.77 g Hepes and 2.2 g $NaHCO_3$ in 100 ml of 0.05N NaOH) and RPMI-1640 and kept on ice. Then 150 µl aliquots of this reconstituted collagen solution were placed in each well of 48-well culture plates and immediately incubated at 37°C for 1 hour to allow gel formation. After the gel solidified, HUVEC (1.5×10^4 cells/0.3 ml) were added to well and incubated at 37°C for eight hours. Subsequently, each well was washed in PBS, fixed with 0.5 ml of 100% methanol for 20 minutes, and stained with Giemsa's solution (Merck, Darmstadt, Germany) at room temperature for 30 minutes. The formation of the capillary/tube-like networks were examined by using a phase-contrast microscope equipped with a Sony CCD video camera and evaluated by KS400 imaging system, release 2.0 (Carl Zeiss, Oberkocken, Germany). Once determined, the images were captured and exported to Photoshop (Adobe) or PowerPoint (Microsoft) for high-quality printing. All experiments were repeated at least twice. Results are presented as the means ± SD of eight fields derived from two wells.

ILK kinase assay

HUVEC stimulated with 30 ng/ml of rhVEGF for 5 minutes were lysed in a lysis buffer. The lysates were precleaned with protein A-sepharose, and then incubated with anti-ILK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C for 12 hours. After incubation, immune complexes were collected with protein A-sepharose. The immunoprecipitated ILK was incubated with for 30 minutes at 30°C in a total volume of 50 µl of kinase reaction buffer (20 mM Hepes, pH7.0, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM NaF, 1 mM Na_3VO_4, 100 µM ATP) in myelin basic protein (MBP)-coated 96-well plate. Phosphorylation of MBP was detected using anti-phospho-ser/thr (Upstate Biotechnology Inc.), secondary antibody coupled to horseradish peroxidase and chemiluminescent substrate (PIERCE, Rockford, IL). Chemiluminescences were measured using WALLAC 1420 Multilabel Counter. Results are presented as mean ± SD of four wells.

Proliferation assay

Aliquots of medium containing 4.0×10^3 cells were seeded into a collagen-coated 96-well plate. The following day, the medium were replaced with serum-free CS-C medium with or without rhVEGF. The plate was then incubated for 48 hours before adding of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (Wako Pure Chemical Industries Ltd., Osaka, Japan). The plates were read at a wavelength of 450 nm using a TECAN Spectra Thermo (Tecan Group Ltd. Raleigh, NC). Results are presented as the mean ± SD of four wells.

Statistical analysis

All results are expressed as the mean ± SD values. The statistical significance was evaluated by using the paired two-tailed Student's t test. A p-value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using JMP 5.01 software (SAS Institute Inc. Cary, NC).

RESULTS AND DISCUSSION

Formation of a capillary/tube-like network is induced by VEGF in human vascular endothelial cells

In vitro angiogenesis of human vascular endothelial cells was assessed using a capillary/tube-like network formation assay.

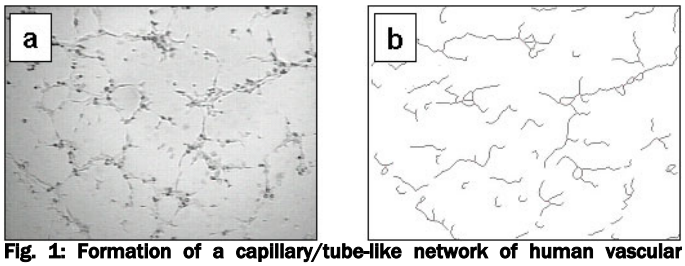

Fig. 1: Formation of a capillary/tube-like network of human vascular endothelial cells stimulated with VEGF. HUVEC were stimulated with 30 ng/ml of VEGF and cultured in a type I collagen gel-coated plate for eight hours at 37°C. The microscopic image was recorded by a CCD video camera and analyzed using image analysis software. a: Microscope image, b: Processed image.

HUVEC that were seeded onto a type I collagen gel matrix in RPMI-1640 medium with 30 ng/ml VEGF displayed formation of capillary/tube-like networks after eight hours of incubation at 37°C (Fig. 1A). Images were

taken using a phase-contrast microscope equipped with a CCD video camera and were evaluated by the imaging system. Figure 1B shows a processed image of capillary networks of VEGF-treated HUVEC. Three angiogenic parameters: capillary length per field, number of capillaries per field, and relative capillary area per field, were considered as measures of *in vitro* capillary/tube-like network formation for assay development. In this study, we used capillary length per field for evaluation of angiogenesis activity. Figure 2 clearly shows that VEGF induced a concentration-dependent (10-100 ng/ml) formation of capillary/tube-like networks of HUVEC. It has also been demonstrated that VEGF induces network formation of HUVEC when reconstituted basement membrane matrigel (Beckton, Dickinson and Company) was used instead of a type I collagen gel matrix (16). The matrigel is a basement membrane-like matrix produced by the Engelbreth-Holm-Swarm (EHS) tumor, and it contains laminin-1, entactin, and type IV collagen (17, 18). This result indicates that the ability of human vascular endothelial cells to form a capillary/tube-like network in response to VEGF stimulation may not dependent on the type of matrix.

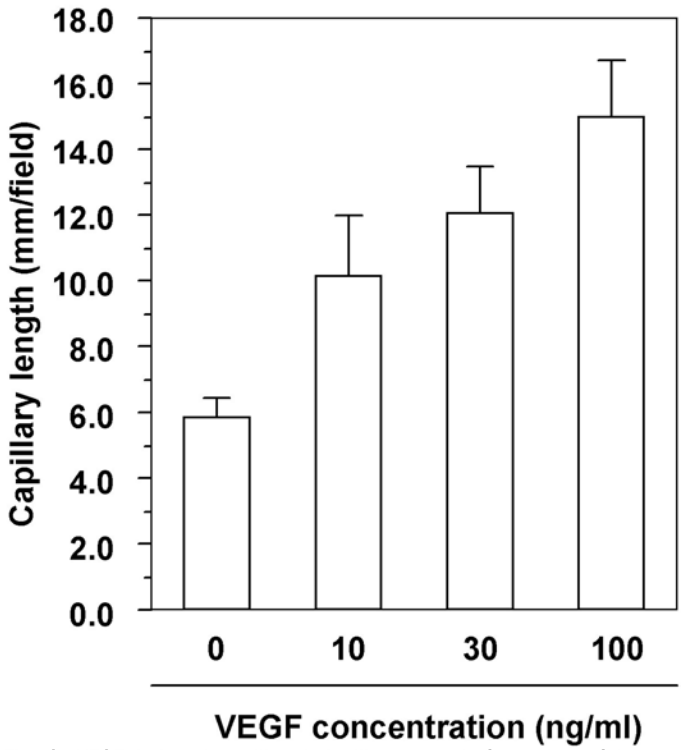

Fig. 2: VEGF induced capillary/tube-like network formation of human vascular endothelial cells in a concentration-dependent fashion. HUVEC (1.5×10^4 cells/well) were incubated in a type I collagen gel-coated plate with the indicated concentration of VEGF at 37°C for eight hours. The microscopic image was recorded by a CCD video camera and analyzed using image analysis software. The results represent the mean ± SD values.

Regression of capillary/tube-like network formation by ILK inhibition using a dominant negative ILK

Recent studies have demonstrated that cellular responses to various growth factors, including VEGF and platelet-derived growth factor (PDGF), are modulated by the activation of integrin adhesion receptors. Furthermore, the cytoplasmic regions of integrins associate with catalytic signaling molecules such as ILK and focal adhesion kinase (FAK). Our next experiment was designated to evaluate whether VEGF-induced formation of capillary/tube-like networks in a type I collagen gel matrix was mediated by ILK. A potent dominant negative, kinase-deficient form of ILK (ILK-KD) contains a point mutation substituting a lysine for the glutamic acid at position 359 in the kinase domain (19). As shown Figure 3, ILK-KD could inhibit ILK kinase activity as a dominant negative. The N-terminal region of ILK contains a pleckstrin homology (PH)-like domain that probably binds a phosphatidylinositol-3, 4, 5-triphosphate, which is a lipid product of phosphatidylinositol 3-kinase (PI 3-kinase). This domain is likely to participate in the regulation of ILK kinase activity (20). A selective inhibitor of PI 3-kinase, LY294002, also inhibited kinase activity of ILK (Fig. 3).

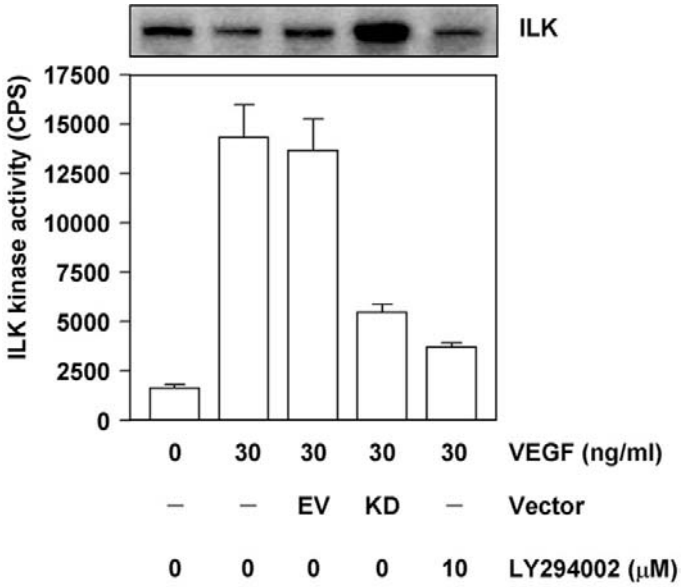

Fig. 3: Inhibition of ILK kinase activity by ILK-KD or LY294002 in HUVEC. The cells were stimulated with 30 ng/ml VEGF for 5 minutes, and then ILK activity was measured by ILK kinase assay. Anti-ILK blot was prepared from the same immunoprecipitates used for the kinase assay (top panel). Bars represent the mean ± SD values. EV, empty vector; KD, ILK-KD.

The VEGF-triggered capillary/tube-like network formation was inhibited by transfection with a vector encoding ILK-KD (Fig. 4).

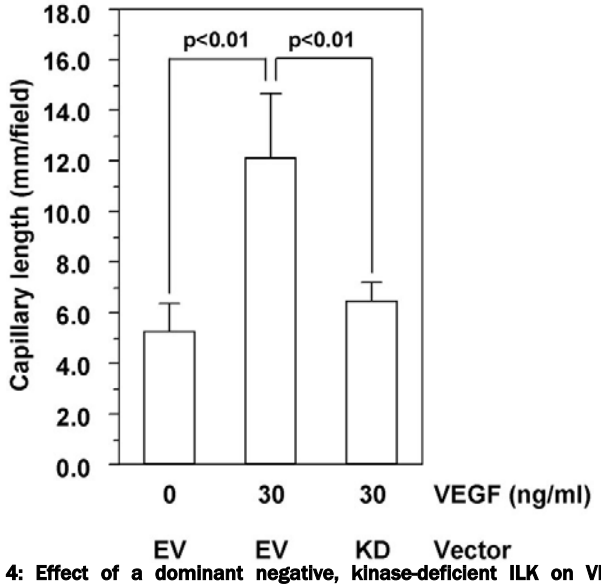

Fig. 4: Effect of a dominant negative, kinase-deficient ILK on VEGF-induced capillary/tube-like network formation of human vascular endothelial cells. HUVEC were transiently transfected with the indicated plasmids (0.5 μg/ml) 18 hours prior to VEGF stimulation. The cells were incubated in a type I collagen-coated plate for eight hours at 37°C with or without 30 ng/ml VEGF. Bars indicate as the mean ± SD values. The statistical significance is given for the difference between the empty vector-transfected groups plus and minus VEGF stimulation, and for the difference between VEGF-stimulated cells transfected with an empty vector or with a vector encoding ILK-KD, respectively. EV; empty vector, KD; ILK-KD.

Moreover, LY294002 also suppressed capillary/tube-like network formation of HUVEC stimulated with VEGF through inhibition of ILK kinase (Fig. 5).

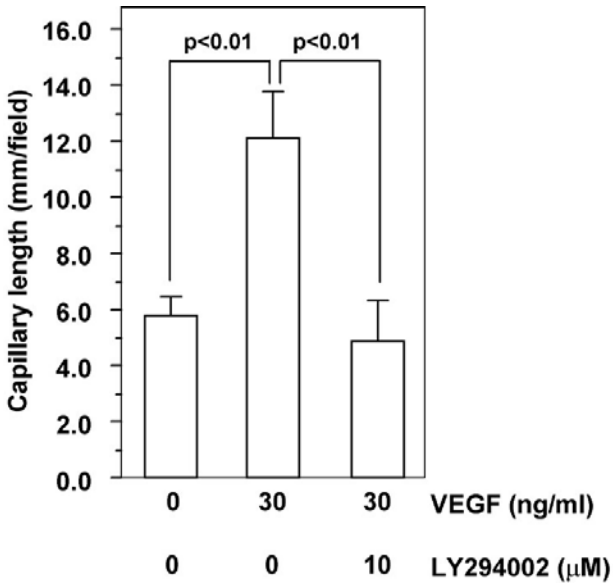

Fig. 5: Effect of a LY294002 on VEGF-induced capillary/tube-like network formation of human vascular endothelial cells. LY294002 was added 30 minutes before VEGF stimulation. The cells were incubated in a type I collagen-coated plate for eight hours at 37°C with or without 30 ng/ml VEGF. Bars indicate as the mean ± SD values.

Since it has been demonstrated that VEGF is an inducer for proliferation of vascular endothelial cells (21), and we next examined the effect on ILK-KD on HUVEC proliferation induced by VEGF. The cells were cultured in the absence or presence of 30 ng/ml VEGF for 48 hours, and then viable cells were measured by WST assay. Transfection of ILK-KD partially inhibited VEGF-stimulated proliferation of HUVEC (Fig. 6).

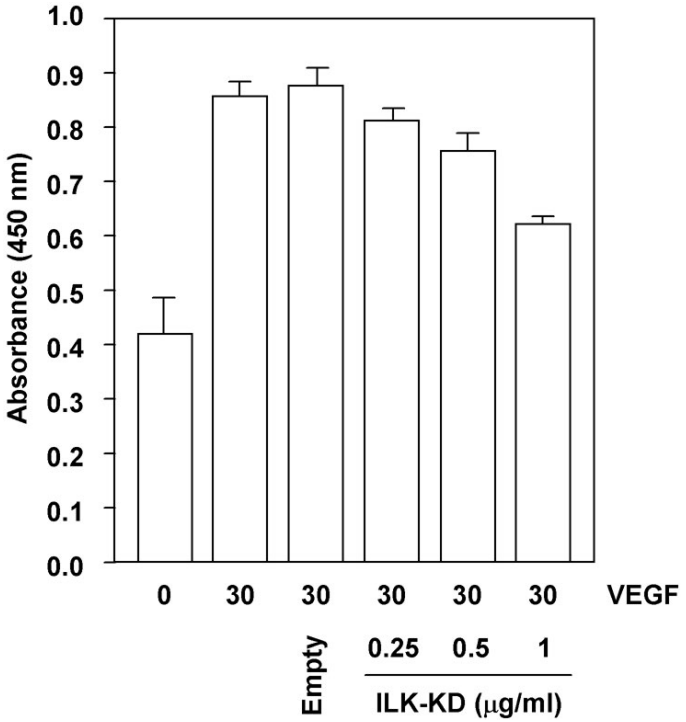

Fig. 6: Effect of ILK-KD on HUVEC proliferation stimulated with VEGF. HUVEC were transiently transfected with the indicated vectors and incubated for 18 hours. The cells were cultured in the absence or presence of VEGF for 48 hours. Viable cells were measured by WST assay. The results represent the mean ± SD values.

Our data indicated that ILK and PI 3-kinase are involved in VEGF signaling pathways mediating capillary/tube-like network formation of human vascular endothelial cells.

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PROTOCOLS

Protocol 1: Cell culture

- Culture human umbilical vein endothelial cells (HUVEC) in collagen-coated flasks in CS-C medium supplement with 10% FBS.
- For capillary/tube-like structure formation assay, use RPMI-1640 instead of CS-C medium.

Protocol 2: Reconstitution of type I collagen gel solution

- Place a sterile tube on ice.
- Add eight volumes of 0.3% cold Cellmatrix type I-A solution to the tube.
- Add one volume of reconstitution buffer (4.77 g Hepes and 2.2 g $NaHCO_3$ in 100 ml of 0.05N NaOH) dropwise while swirling the tube. Do not shake vigorously.
- Add one volume of cold medium and mix well. For serum-supplemented culture, routinely add one volume of cold FBS.

Protocol 3: Formation of a type I collagen gel layer

- Transfer 150 μl cold reconstituted type I collagen gel solution into each well of 48-well culture plates. Use a pipette with a wide mouth since the collagen solution is viscous.
- Place the plate in a 37°C humidified 5% CO_2 incubator and wait for 1 hour to allow gel formation.

Protocol 4: Capillary/tube-like network formation assay

- Collect human umbilical vein endothelial cells.
- Suspend the pelleted cells in medium and wash twice.
- Plate the cells (300 μl of suspended cells with or without angiogenic factor) on type I collagen gel layer in each well.
- Incubate at 37 °C for 8 hours.
- Remove medium and wash with PBS twice.
- Fix with 0.5 ml of methanol and incubate for 20 minutes at room temperature.
- After removal of the methanol, stain the cells with Giemsa's solution (1:20) and left to incubate at room temperature for 30 minutes.
- Wash the dishes twice with H_2O.
- Record the microscope images with a CCD video camera and analyzed with an image analysis software.
- After analysis, capture the images and export to Photoshop (Adobe) or PowerPoint (Microsoft) for high-quality printing.