Biotechnol. Bioprocess Eng. **2002**, 7: 178-184

Characterization of the KG1a Cell Line for Use in a Cell Migration Based Screening Assay

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Abstract High-throughput screening has become a popular method used to identify new "leads" for potentially therapeutic compounds. Further screening of these lead compounds is typically done with secondary assays which may utilize living, functioning cells as screening tools. A problem (or benefit) with these cell-based assays is that living cells are very sensitive to their environment. We have been interested in the process of stem cell migration and how it relates to the cellular therapy of bone marrow transplantation. In this study we describe a secondary, cell-based assay for screening the effects of various *in-vitro* conditions on Immature Hematopoietic Cell (IHC) migration. Our results have revealed many subtle factors, such as the cell's adhesive characteristics, or the effect of a culture's growth phase, that need to be accounted for in a screening protocol. Finally, we show that exponentially growing KG1a cells (a human IHC cell line) were 10 times more motile than those in the lag or stationary phases. These data strongly suggest that KG1a cells secrete a chemokinetic factor during the exponential growth phase of a culture.

Keywords: cell motility, hematopoietic stem cells, time lapse microscopy, cell migration

INTRODUCTION

To study the process of cell motion in response to chemokinetic compounds *in vitro*, one must be able to simulate *in vivo* conditions during the assay. Some cell types need cytokines or extracellular matrix to survive *in vitro* but in general, the major requirements for maintaining the cells are temperature, pH, osmolarity, and nutrient concentrations [1]. For cell culture work, meeting these requirements usually involve placing the cells in culture medium that is buffered to control pH, and maintaining the temperature at *in vivo* levels using incubators. Microscopy and imaging technology are continuously improving and it is becoming commonplace to study cell migration by continuously monitoring the movements of individual cells in a "time-lapse" mode [2, 3]. Continuous monitoring therefore requires recreating the conditions inside the incubator on the microscope stage. Various systems have been developed to do this that usually involve warm air blowers or conductive heat sources with CO_2 gas perfusion systems [2].

We experimented with warm air blowers and commercial stage incubators from Bioptics (Butler, PA, USA) and 20/20 Technology Inc. (Wilmington, NC, USA) in our initial attempts at measuring Immature Hematopoietic Cell (IHC) motility via time-lapse cell tracking. What we found was that IHCs do not adhere firmly to the substrate during migration. As a result, temperature gradients induced by uneven heating of the top and bottom surfaces of the media created interesting flow patterns within the culture well, which corrupted cell track data. Unlike adherent cell types such as fibroblasts or endothelial cells, IHCs are susceptible to these environmental disturbances due to their weak adhesive properties. These problems led us to the development of an Automated Time Lapse Microscope System (ATLMS) and an assay for measuring the migration characteristics of loosely adherent cells [4].

The cell migration assay system we developed had to meet the following requirements. First, it needed to maintain the cells in a stable environment (temperature, pH and osmolarity) for up to a week or more. This long time period was driven by the desire to monitor cell division and colony formation as well as cell motion with the same system. Second, it needed to be automated so that many different cultures and conditions could be screened during one experiment. Standard, plastic, multi-well tissue culture plates were selected to culture the cells in due to the wide selection of sizes, shapes and coatings commercially available. Finally, continuous monitoring of the cells at any time interval is necessary so that cellular processes occurring over time scales from seconds to weeks could be studied. The solution that evolved which satisfied all these constraints was to completely enclose an automated microscope within a plexiglass box maintained at 37°C with a warm air blower and 5% CO_2 using an infra-red sensor and custom built controller.

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Using the ATLMS we were able to measure and characterize the motility of the KG1a IHC line under a variety of *in vitro* conditions. Preliminary experiments were performed to identify the controls necessary for the inclined migration assay. In the process, we discovered a growth phase effect on motility and traced the cause to a soluble factor in the media.

MATERIALS AND METHODS

Cells

The successful migration of IV infused hematopoietic stem cells (HSCs) into the bone marrow cavity is a critical step in HSC transplantation. This process is poorly understood in cellular and molecular terms and is currently subject to intense investigation. To facilitate study of HSC migration, the IHC line KG1a was selected as a model cell line for our HSC migration studies.

The acute myelogenous leukemia cell line, KG1a, was first isolated in 1980 [5]. A prominent phenotypic abnormality of these cells is their inability to differentiate into functionally mature cells, causing them to remain in an early or primitive state of development [6]. Because of their primitive nature, KG1a cells were used in a strategy to develop antibodies that recognized IHCs [7]. The result was the first antibody against the cell surface marker CD34, which has since become the marker of choice in progenitor cell selection for hematopoietic studies as well as enrichment protocols for stem cell therapies [8]. KG1a cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were received cryopreserved and then rapidly thawed and suspended in IMDM (GibcoBRL, Grand Island, NY, USA) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 20% heat inactivated fetal bovine serum (GibcoBRL). A consistent serum lot was used throughout these experiments to eliminate lot-to-lot variability effects. The cell line was maintained in an incubator at 37°C, 95% humidity and 5% CO_2. Passage was performed every 3 to 4 days, by diluting the cells approximately 1:5.

Image Collection and Processing

Cells were imaged using an inverted Eclipse TE300 microscope (Nikon Inc., Melville, NY, USA) equipped with a 20X Hoffman Modulation Contrast (Modulation Optics Inc., Greenvale, NY, USA) objective. Digitized images were acquired with a cooled Sensys CCD camera (Photometrics Inc., Tucson, AZ, USA) mounted on the side port of the microscope. Utilizing the Sensys and a 20X objective gave a resolution of approximately 0.35 μm/pixel. Images were then sent directly to a SGI O_2 workstation (Silicon Graphics, Mountain View, CA, USA) for storage. In addition to the camera, the motorized microscope stage, z-focus motor (Ludl, Hawthorne, NY, USA), and Uniblitz transmitted light shutter (Vincent Associates, Rochester, NY, USA) were also controlled from the O_2 workstation. All acquisition and processing functions were performed by Isee™ software (Inovision Corp., Durham, NC, USA).

Inclined Migration Assay Configuration

Fluid motion due to temperature gradients within the culture wells was significantly reduced by the construction of the ATLMS. However, subsequent experiments indicated that there might be some small second order effects, such as acoustic vibrations from the fan, that were adding a small bias to the cell track data. To eliminate these effects, we modified a technique first used by Strobel *et al.* [9], in which the entire culture surface was inclined at a 15° angle, requiring the motile cells to move against the force of gravity. Only cells that are in an active, migratory state will adhere firmly enough to move up the incline. We chose a shallower angle of 7° due to geometric constraints within the ATLMS. KG1a cells were plated at a density of 1.33×10^4 cells/mL under various experimental conditions on 96 well plates (Beckton Dickenson, Bedford, MA, USA). Immediately after plating, sterile water was added to the unused wells to reduce evaporation. The lid was then replaced and sealed with masking tape, which allowed CO_2 to pass through. Using this technique we were able to control evaporation to less than 20% over an 8 day time period. Correct levels of pH were confirmed by with a pH meter and by the change in the color of the phenol red containing medium. Viability was routinely measured at over 95% by adding propidium iodide (Boehringer Mannheim, Indianapolis, IN, USA) to the wells after the experiment, confirming that the ATLMS was functioning properly. After sealing, the plates were placed in an incubator for approximately 2 to 4 h, tilted at an 80° angle to allow the cells to settle down to a "starting line" at the lower end of the well, Fig. 1(a).

Data Collection and Analysis

After settling, the plate was transferred to the ATLMS. Each well was individually focused and the X, Y, Z position of the stage was programmed to take a strip of four adjacent images starting from the bottom center of the well and moving vertically upwards. Once the experiment began, time intervals between images were typically on the order of 3 h with a total duration of 15 h. During the first 3 h of the experiment, the starting line would gradually move upwards due to an "avalanche" of cells sliding down after having settled high against the wall. For this reason we only counted cells that had migrated at least 50 μm from the original starting line. This distance is somewhat arbitrary but it was chosen large enough to avoid the avalanche effect, yet small enough so that a statistically significant number of cells could be counted as having migrated. Boxes were drawn on the images and the number of cells that had migrated between 50 and 850 μm from the starting

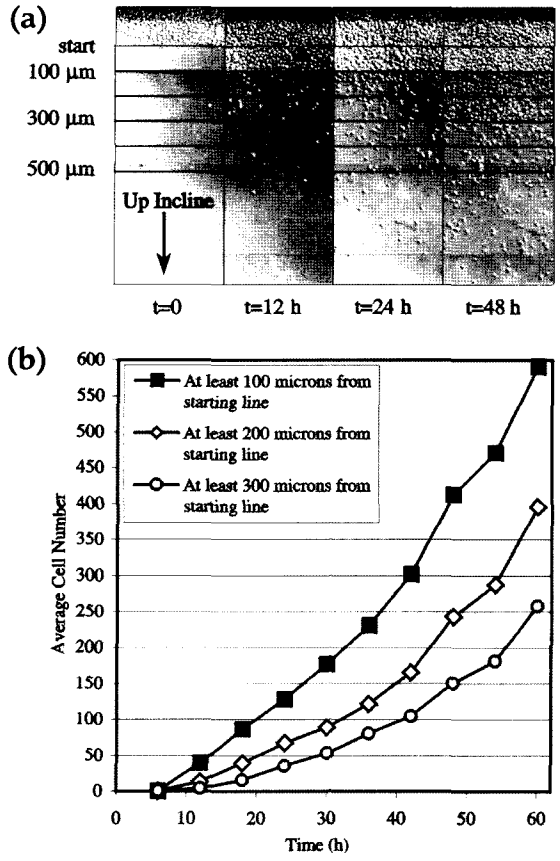

Fig. 1. (a) KG1a Cells in the Inclined Migration Assay. After approximately 2 h at a high (80 deg) angle, the cells have settled down to the starting line, t=0. The plate is then placed on the inclined microscope at an incline of approximately 7 degrees. Mosaics of the well are taken at various time intervals and the number of cells migrating uphill can be counted as a function of time and distance from the starting line. (b) Effects of Cell Division on Motility Data. In this long duration experiment, mosaics were made of the well every 3 h and the number of migrating cells were counted and plotted as a function of time and distance migrated.

line was tabulated. Occasionally, a few cells did not settle down to the starting line so they were subtracted from the total. The data were then plotted as the average value from 3 to 6 identical wells. This average value after 15 h, then became our metric for comparing the motility of KG1a under a variety of experimental conditions.

Quantification of cell migration in terms of parameters such as the random motility coefficient μ, and chemotaxis coefficient χ has been the objective of many leukocyte migration studies. While computation of these terms is certainly possible using our system, we elected to use the "cells migrating past 50 microns" metric since it was simple and sufficient for evaluating our differences between experimental variables.

RESULTS AND DISCUSSION

The process of adapting the inclined migration assay for the purposes of this study necessitated performing several control-type experiments to characterize and optimize the assay. These results will now be described.

Experiment Length

In the process of functional testing of the ATLMS, experiments of prolonged duration were performed with the KG1a cell line. These experiments were not possible when working with mature, primary cells such as neutrophils due to their short lifetime (of the order of hours). A cell line is capable of dividing indefinitely so the KG1a cells were a natural choice for testing the viability of hematopoietic cells in the ATLMS over the time scales (on the order of days) required for cell division experiments. For measurements of the migration process, data were tabulated every 3 h for 60 h and plotted as a function of time and distance migrated as shown in Fig. 1(b). Due to the prolonged duration of this experiment, the density of cells near the starting line became very high and nearly uncountable at later time points. For this reason, the counting criteria of 50 μm was no longer appropriate. One, two and three hundred microns were the distances chosen and the average number of cells migrating past these distances as a function of time was tabulated.

One may also notice that the slope of the curves in Fig. 1(b) is generally linear until about 30 h at 100 μm, when the slope began to increase. When plotted on a semi log scale, the curve appears to be a straight line (not shown), indicating an exponential increase in the cell density. The density increase observed in the inclined migration data was in part due to migration of the KG1a cells, but the increase was faster than could be attributed to migration alone. Measurement of migration and division become coupled after a period of time since the cell count is influenced by dividing cells and the flux of migrating cells. The doubling time for KG1a is roughly 24 h, calculated by monitoring the batch culture density, so detection of noticeable division effects in this assay after 30-35 h seems reasonable. Interestingly, the onset of the exponential upturn in cell density occurred later at greater distances. The delay is due to the time it takes for the cells to migrate out to a given distance after which they begin to divide.

Substrate Effects

The inclined migration assay was also used to characterize effects due to the density of extracellular matrix components on the migration surface Substrate effects on migration have been described by models that account for receptor-ligand affinity as well as substrate density and predict maximum speeds under optimal conditions [10-12]. A 96 well plate was coated with human fibronectin at different densities following the manufacturer's (Becton Dickinson Labware) protocol

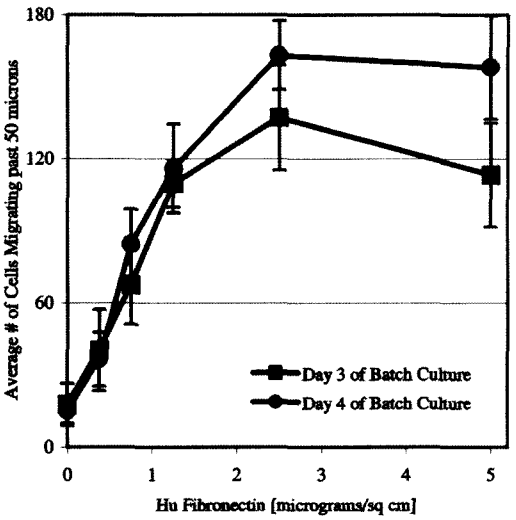

Fig. 2. KG1a Migration vs. Human Fibronectin Coating Density. Human fibronectin was coated at different densities on a 96 well plate. As the substrate density increases, the adhesion of the cells improves allowing them to migrate better. This experiment was performed on 2 consecutive days using cells from the same batch culture to ensure that the measurements were made when the cell motility was highest.

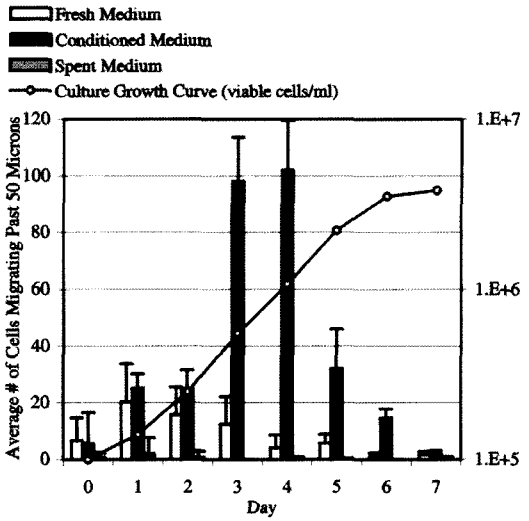

Fig. 3. KG1a Motility vs. Growth Phase. KG1a cells reach their peak motility when they are growing in their mid exponential phase. This may seem obvious but the most interesting result is the difference in motility between conditioned and fresh media. The batch culture's growth curve is plotted along the right y-axis.

As the substrate density increased, the adhesion improved and the cells were able to migrate farther over the 15 h duration of the experiment as shown in Fig. 2. This experiment was performed on 2 consecutive days to ensure that the measurements were made when the cell motility was highest, as dictated by the results of the growth phase experiment discussed below. The trend shown is in general agreement with results published by DiMilla *et al.* [10]. although the surface concentrations differ. A likely explanation is that our coating densities were calculated based on the protocol provided by the manufacturer whereas DiMilla *et al.* actually measured the adsorbed concentration. From these results we concluded that KG1a migration was enhanced on a fibronectin substrate, enabling clearer distinction between experimental variables. Future experiments therefore utilized consistent lots of precoated (human fibronectin) 96-well plates (Becton Dickinson Labware).

Growth Phase Experiment

Early motility experiments with these cells in the inclined migration assay showed inconsistent results. One day the cells would migrate and when the experiment was repeated a few days later, the motility was greatly diminished. To identify the cause, a lengthy experiment was performed. On day 0, cells from a batch culture in the exponential growth phase were inoculated at 1.0×10^5 cells/mL into T-75 flasks containing fresh medium (IMDM with 20% FBS) and allowed to grow for 8 days without passing. The growth curve of this culture is shown in Fig. 3. Each day a sample was removed from the culture and its motility was measured using the inclined migration assay. The plating density for the inclined migration assay was typically 1.3×10^4 cells/mL so a dilution was required to achieve the appropriate cell concentration.

Three experimental conditions were evaluated: 1) the dilution was performed with fresh media, 2) the dilution was performed with "spent" media (media from a similar culture that had reached its stationary phase), and 3) the dilution was performed with conditioned media from the original batch culture, harvested and filtered at the same time each day as the cell sample. These were just simple dilutions of the cells, washing, staining or centrifugation was not performed so as to minimize the perturbations to the cells.

Two independent batch cultures were created on day 0. Each day, 3 duplicate wells for the 3 different media, were evaluated for a total of 18 wells each day. The results from this experiment are shown in Fig. 3. As we can see, the measured motility of the cells changes as the culture grows, reaching a peak of over 100 cells migrating past 50 microns during the mid exponential phase before declining. This effect is substantial, with the conditioned media outperforming the fresh media by a factor of 10. Motility was non existent when using the spent medium.

Conditioned Media Experiments

The growth phase effect finding spawned a series of new experiments based on the hypothesis that autocrine chemokines were present. First, a culture was

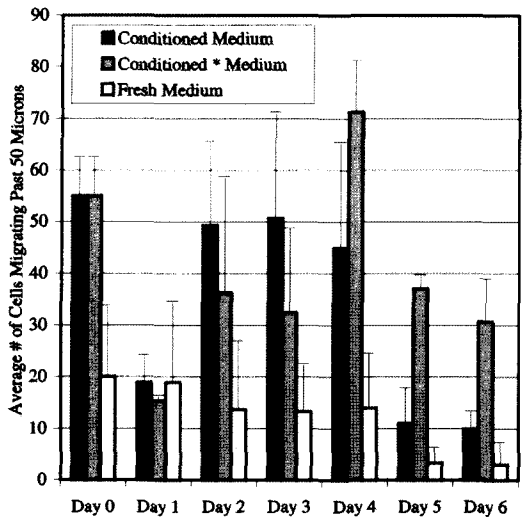

Fig. 4. KG1a Motility vs. Growth Phase with C*. This experiment is similar to the experiment described in Fig. 3, except the spent media has been replaced with conditioned media harvested from an identical culture in its exponential phase. As we can see, the C* media maintains motility well into the stationary phase (day 6) of the culture. The difference at day 0 is due to the definition of the conditioned media. When the batch culture was started, the inoculum was in day 3 of its cycle therefore, the conditioned media was from that culture and shows increased motility as expected.

prepared to produce a large amount of conditioned media. This media (C*) was then harvested on day 3 of the culture. The growth phase experiment was then repeated and the results are shown in Fig. 4. The significant feature of these data is that toward the end of the experiment, the C* medium maintains the motility of the cells even as the culture enters the stationary phase, while the fresh medium does not. By this time, the conditioned medium was approaching the composition of the spent medium so a motility decline was expected.

Of the many different cell migration assays described in the literature, the Boyden chamber assay [13] has been the most popular for studies of IHC chemotaxis [14]. Whereas, the others like the under agarose assay [15,16] have been used more extensively with neutrophils. Since the Boyden chamber is a qualitative endpoint assay which provides little information as to the physical migration processes, additional studies of IHCs are clearly needed.

Our objective in this study was to characterize the migratory processes of IHCs. What we found was that: 1) KG1a cells (as well as primary $CD34^+$ cells, data not shown) do not migrate the same way that mature, adherent cells such as fibroblasts do, 2) because of their loosely adherent characteristics, we needed to engineer an assay system that enabled us to measure differences in migration parameters, 3) migration of KG1a cells is sensitive to *in vitro* conditions such as substrate density, 4) once these conditions were characterized, migratory differences due to growth phase were detected, and 5) conditioned media experiments then suggested that an autocrine chemokine may be secreted when KG1a cells are growing exponentially. To measure migration differences due to the above effects, a population based inclined migration assay [9] was described along with a detailed protocol for experiments with a model IHC cell line, KG1a. In the process of developing the assay protocol, several interesting discoveries were made about the characteristics of KG1a migration. These findings further illustrate the complex nature of the migration process and the difficulties associated with *in vitro* measurements.

When cells are seeded, they usually progress through a characteristic growth pattern of lag phase, exponential phase, and stationary phase. Data from the growth phase experiment show that motility is a strong function of the growth phase of KG1a. However, this is not the complete story. By using the different experimental media conditions as described, additional insight can be gained. The spent media shows zero movement, which is not surprising since it is likely that the nutrients are depleted and metabolites have built up. The fresh media shows very little motility as well, but the conditioned media shows motility that increases as the culture matures and peaks when the culture is in its mid exponential phase before declining. This suggests that the KG1a cells may be secreting a soluble chemokinetic factor(s) into the media.

An obvious next step was to see if this factor could be isolated. Isolation would require performing the experiment in serum free media (the same IMDM without the addition of fetal bovine serum) so that any newly secreted protein could be identified without the many serum proteins in the background. Attempts to adapt the KG1a cells to serum free medium were not successful and resulted in poor viability. Furthermore, when the cells were diluted in the serum free media, they were transformed in a way that made them tightly adhere to the substrate, preventing them from settling down to the starting line.

In the conditioned media experiment, the expectation was that C* would produce a high level of motility throughout the 8 day experiment. That was not the case in the beginning of the experiment but after the motility peak, C* kept the cells motile well into the stationary phase. If the cause for this enhanced performance was simply that C* was "fresher" than C, and contained more nutrients, then the cells in the fresh media should also perform better, and if the chemokine was the only reason for motility then the cells in the early phase of the culture should also be stimulated.

Results from these experiments strongly suggest that a soluble factor is responsible for the measured increase in motility. However, the explanation will likely be more complicated and probably involves the interaction of several factors. Cells are known to adapt in many different ways to varying culture conditions. For instance, Frame and Hu [17] have reported that cell mass changes significantly during exponential growth phase.

Cell mass peaked at the early exponential phase and decreased as the culture progressed, while the ratio of protein to cell mass (dry cell weight) was constant. Since cells in early exponential phase are bigger and have more mass including protein, they may be ready to secrete autocrine factors. Perhaps an explanation for the C* effect would be that in the early stages of the culture, the cells are not sensitive to the soluble factor and then as the culture reaches the end of its exponential phase, production is shut down and the remaining amount is consumed as the cells enter the stationary phase. Taken together, these findings suggest that new experiments designed to identify differences in gene expression over the different growth phases may lead to a better understanding of the genetic circuits involved in cell migration and division.

CONCLUSION

In a critique on methods used to assess leukocyte behavior by Lackie *et al.* [18], they points out that assay systems can be divided into two types: reductionist and realistic. In a reductionist assay, the hope is to control all variables but one and assume that that one was responsible for the cellular behavior being measured. A realistic assay intends to mimic the *in vivo* environment without inducing artifacts, and quantify the behavior of the cells. The difficulty of this task became apparent when confronted with the problem of controlling for miniscule amounts of fluid motion within the culture wells. Using the ATLMS, one can create as realistic an environment as possible for the cells *in vitro* and sustain it for long periods of time. Reductionist type experimental variables such as chemokine gradients for the study of chemotaxis or extracellular matrix composition can also be introduced into the experiment. The many new reductionist type variables revealed by this study suggest that one use caution when comparing quantitative results between seemingly identical repeats of the same experiment. We have shown that it is very difficult to recreate the exact realistic environment each time due to subtle details such as growth phase. Furthermore, different cell types have unique properties and the methods and models that work for one cell type cannot be applied universally. Only by working with a cell type and understanding its characteristics such as growth kinetics and migratory mechanisms can one avoid potentially misleading conclusions. Collectively, these experiments show just how sensitive KG1a cells are to their environment. This in turn has implications as to the effects that cell separation processing using flow cytometry or magnetic beads may have on the motility of transplanted primary stem cells. These findings can have profound implications when using cell-based assays in the drug discovery process where false positives or negatives can result in wasted time and resources, or the passing over of a potentially lucrative therapeutic agent.

Acknowledgements We would like to acknowledge the assistance of Brian Kidd for his help in data acquisition and analysis. This work was supported by public health service grants HL 59234 and HL 60938 from the NIH.

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[Received March 6, 2002; accepted May 27, 2002]