Video Article

Assessment of Endothelial Cell Migration After Exposure to Toxic Chemicals

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Abstract

Exposure to chemical substances (including alkylating chemical warfare agents like sulfur and nitrogen mustards) cause a plethora of clinical symptoms including wound healing disorder. The physiological process of wound healing is highly complex. The formation of granulation tissue is a key step in this process resulting in a preliminary wound closure and providing a network of new capillary blood vessels – either through vasculogenesis (novel formation) or angiogenesis (sprouting of existing vessels). Both vasculo- and angiogenesis require functional, directed migration of endothelial cells. Thus, investigation of early endothelial cell (EEC) migration is important to understand the pathophysiology of chemical induced wound healing disorders and to potentially identify novel strategies for therapeutic intervention.

We assessed impaired wound healing after alkylating agent exposure and tested potential candidate compounds for treatment. We used a set of techniques outlined in this protocol. A modified Boyden chamber to quantitatively investigate chemokinesis of EEC is described. Moreover, the use of the wound healing assay in combination with track analysis to qualitatively assess migration is illustrated. Finally, we demonstrate the use of the fluorescent dye TMRM for the investigation of mitochondrial membrane potential to identify underlying mechanisms of disturbed cell migration. The following protocol describes basic techniques that have been adapted for the investigation of EEC.

Video Link

The video component of this article can be found at http://www.jove.com/video/52768/

Introduction

Cell migration is important in many physiological and pathophysiological processes including development, various diseases, and wound healing after skin injury.

Following skin injury, inflammation removes damaged or necrotic tissue and granulation drives preliminary wound closure and allows formation of a network of new capillaries through vasculogenesis (novel formation) or angiogenesis (sprouting of existing vesicles)[1-3]. Both vasculo- and angiogenesis require migration of endothelial cells. The growing network of blood vessels is essential to transport oxygen and nutrients to proliferating keratinocytes which ultimately undergo keratinization, form a new epithelium and provide wound closure.

Impaired migration of endothelial cells is an underlying cause of wound healing disorder[4,5]. Thus, methods to assess migration of early endothelial cells are required to explore the pathophysiology of cell migration disorders and to identify novel strategies for therapeutic intervention.

Dermal exposure to alkylating agents (*e.g.,* sulfur and nitrogen mustards) causes wound healing disorder[6]. Such compounds were used as chemical warfare agents in several conflicts in the 20th century and remain reason for strong concern due to existing stockpiles in politically unstable regions and the relatively simple synthesis. Although sulfur mustard was first synthesized in 1822, the molecular and clinical pathology of SM exposure is not understood in detail and no antidote for SM exposure has been identified.

Several studies have been conducted to understand and to model impaired wound healing after SM exposure and to test for potential candidate compounds capable of reserving that effect. Schmidt *et al.* (2009) tested the effect of chlorambucil, an alkylating compound with properties similar to SM in mouse embryoid body models and found a dramatic, sometimes more than 99% reduction in vessel formation[7]. This adverse effect was most pronounced at a stage of development which, under physiological conditions, is dominated by the proliferation and migration of vascular endothelial precursor cells. Thus, these cells were identified to be particularly sensitive to alkylating agents. Steinritz *et al.* (2010) tested scavengers of reactive oxygen species (ROS), in particular, N-acetylcysteine (NAC) and alpha linolenic acid (ALA) for their ability to reduce SM toxicity in mouse embryoid body models and in particular, to restore vessel formation[8]. Temporary protective effects were observed, indicating that excessive ROS formation was likely to contribute to the adverse effects of SM on wound healing. These effects were not permanent and the two candidate compounds may not be capable of restoring vessel formation and wound healing in the long term[8]. However, those experiments

were conducted in a complex 3D model which did allow investigation of cell migration. Thus, we subsequently tested NAC and ALA for beneficial effects on cell migration of EEC that have a key role in the process of vessel formation[9].

Moreover, there is evidence that cell polarity is required for cell migration. Mitochondrial dysfunction leading to ROS formation was shown to impair cell polarity and may thus adversely affect cell migration. Therefore, live cell imaging with regard to mitochondrial function was performed and the effects of ROS scavengers were examined. The following protocol describes general requirements for the cultivation of EEC, the Boyden chamber assay, the wound healing assay including cell tracking analysis and the use of TMRM for assessment of mitochondrial function in detail. Important aspects of experimental protocols for EEC cultivation and migration are highlighted.

Protocol

The following protocol describes techniques for the investigation of early endothelial cell migration. The proper cultivation of vascular endothelial cells requires pre-coating of cell culture flasks with gelatin to ensure proper proliferation and maintenance of an endothelial phenotype.

1. Pre-coating of Cell Culture Flasks

1. Dissolve gelatin in 0.1 M PBS to a final concentration of 0.1%.
2. Autoclave the solution with parameters for liquid autoclaving (for details see instructions of the specific autoclave).
3. Add sufficient volume of the autoclaved gelatin solution to a sterile cell culture flask (*e.g.,* at least 5 ml for a T25 cell culture flask).
4. Transfer the flasks into an incubator (37° C, 5% CO_2 is not required but does not interfere) for at least 30 min.
5. Remove the remaining gelatin solution. Use the pre-coated flasks subsequently (see cell cultivation) or store under sterile conditions until use.

2. Cell Cultivation of Early Endothelial Cells

Note: Embryonic stem cell derived early endothelial cells (EEC) were obtained from differentiated murine embryoid bodies by magnetic-activated cell sorting of the PECAM-1 positive cell fraction as described earlier[10,11].

1. Culture EEC on gelatin-coated cell culture dishes in DMEM supplemented with 15% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, 200 µM L-glutamine, 100 µM ß-mercaptoethanol and 1% non-essential amino acids. Handle cells under sterile conditions. Cultivate the cells with 5% CO_2 at 37 °C and 95% humidity until sub-confluence (max. 80%).
2. At sub-confluence, split the cells at a 1:5 ratio. Note: Detachment of endothelial cells is a critical step.
    1. Harvest the cells with RT accutase. Remove the media, rinse with PBS and add 1 ml of accutase per 25 cm^2.
    2. Incubate the flask at RT for 2-10 min until the cells have detached. Disperse the cells and transfer them to the desired application. Note: A chemical neutralization of the accutase is not required as it takes place when the seeded cells are stored in the incubator at 37 °C. However, accutase activity can also be decreased by adding DMEM containing FCS.

3. Boyden Chamber

Note: Boyden chamber assays are performed by using light-opaque polyethylene terephthalate insert systems with 8 µm pore size.

1. Pre-coat the filter inserts (that fit inside cell culture wells thus creating a Boyden chamber) by adding 500 µl of 0.1% gelatin dissolved in 0.1 M PBS for at least 30 min.
2. If cells are to be exposed to toxic chemicals, expose according to the specific experimental design before cell harvesting. Note: Cells were exposed to 12.5 µg chlorambucil/ml DMEM for 24 hr. With regard to the specific experimental design, instructions may vary.
3. Harvest EEC and determine the cell number by using a counting cell chamber. Note: Automatic counting devices can be used, but should be used with caution: manual cell counting is more accurate and is highly recommended.
4. Add 500 µl cell culture medium into the lower chamber compartment of the Boyden Chamber.
5. Add exactly 10^4 EEC in 500 µl cell culture medium per filter insert in the upper chamber compartment. Eliminate bubbles.
6. Incubate the filter inserts in the incubator for exactly 8 hr.
7. Rinse with PBS once and replace the medium with 0.5 ml 4% paraformaldehyde in both compartments for 25 min for cell fixation. Wash the filter extensively but at least 3 times with 0.1 M PBS.
8. Excise the membranes with a scalpel.
9. Mount the membrane between two glass cover slips with mounting medium containing DAPI for nuclear staining. Pay attention to the orientation. Ensure that only cells that have migrated towards the lower compartment side of the membrane are counted.
10. Count cells that have migrated towards the lower compartment side of the membrane with a fluorescence microscope at 400X magnification. Do not confuse membrane pores with migrated cells (**Figure 1A**, **1B**). Investigate a reasonable number of biological replicates (at least 3 biological replicates per condition).

4. Wound Healing Assay

1. Depending on the available equipment, carefully choose the cell culture dishes or plates: When using DIC microscopy, avoid plastic surface based culture dishes or well plates but use glass based devices instead.
   Note: If using phase-contrast microscopy, plastic based dishes can also be used.
2. Cultivate EEC in a suitable cell culture device (*e.g.,* 4 cm glass bottom petri dish suitable for live cell imaging) until 80% confluence. Important: do not cultivate the cells to complete confluence.

3. Scratch the monolayers with sterile 10 µl pipette tips. Push the tip gently without too much pressure onto the dish surface and move it in a straight line smoothly from one side to the other.Wash the cells twice with 0.1 M PBS to remove detached cells.
4. Add a sufficient volume of medium to the culture dish. If applicable, add compounds that should be investigated.
   Note: 1.5 ml medium containing 15 ng/ml alpha linolenic acid was added.
5. Mount the culture dish under a microscope capable of live cell imaging. Ensure 5% CO_2, 37 °C and a humidified atmosphere.
   Note: Humidification is especially important to avoid medium evaporation.
6. Acquire time-lapse images over 24 hr at 10 min intervals. Plan for large file sizes. Note: A resolution of 512 x 512 pixels is usually sufficient; however, we recommend using images of at least 1,024 x 1,024.
7. Measure the gap width at t = 0 hr and at t = 24 hr using the length tool of the software provided with the microscope or use open-source software (*e.g.,* ImageJ). Note: In general specific image acquisition and analysis software is provided by the manufacturer. Therefore, for technical details regarding the use of the software, refer to the manual.

5. Cell Tracking

1. Depending on the available equipment, carefully choose the cell culture dishes or plates: When using DIC microscopy, avoid plastic surface based culture dishes or well plates but use glass based devices instead.
   Note: If using phase-contrast microscopy, plastic based dishes can also be used.
2. Seed 5 x 10^4 EEC in supplemented DMEM in a suitable cell culture device (*e.g.,* 24-multiwell plate) and cultivate the cells with 5% CO_2 at 37 °C and 95% humidity for 1-2 days.
3. When cells have grown up to a 80% confluence, remove the media and culture the cells with new media in the presence of the respective test substances (e.g., 12.5 µg chlorambucil / ml DMEM). Always include control cells (treated with the solvent, *e.g.,* ethanol) at 37 °C for a certain period of time (24 hr, depending on the individual assay system).
4. Mount the culture dish under a microscope capable of live cell imaging (37 °C, 5% CO_2 and 95% humidity). Acquire time-lapse images over 24 hr at predefined intervals. Acquire images at 10 min intervals.
5. Perform manually tracking of EEC by the use of the ImageJ plugin MTrackJ. Choose 10 cells randomly from the field of view and track their movements by adding a data point per point in time using the “Add” command of MTrackJ.

Note: MTrackJ is available for free at [Meijering, \Mtrackj." http://www.imagescience.org/meijering/software/mtrackj/ “] and ImageJ is available at [Rasband, \Imagej." http://rsbweb.nih.gov/ij/ “]. A detailed manual about the MTrackJ plugin is available at “http://www.imagescience.org”.

6. Live Cell Imaging/Assessment of Mitochondrial Membrane Potential

1. Cultivate EEC in a suitable cell culture device (*e.g.,* 4 cm glass bottom petri dish suitable for live cell imaging) up to a 80% confluence. Important: Do not cultivate the cells to complete confluence.
2. If applicable, expose the cells to chemicals. Note: Cells were exposed to 12.5 µg/ml chlorambucil for 24 hr. With regard to the specific experimental design, instructions may vary.
3. Prepare a 10 mM stock solution of tetramethylrhodamine (TMRM) in DMSO. Protect from light. Note: The stock solution can be stored at -20 °C.
4. Dilute the stock solution in cell culture medium to a working solution with a concentration of 10 µM (1:1,000 dilution). Protect from light and use as soon as possible. Note: The working solution can be kept at RT for some time (>1 hr), however, preparation of a fresh working solution is highly recommended.
5. Add 2 µl of the working solution to 1 ml fresh cell culture medium (loading solution).
6. Load the cells by replacing the cell culture medium with the loading solution. Incubate for 15 min at 37 °C, 5% CO_2 and humidified atmosphere (incubator). Caution: Almost all fluorescence indicators are exported by living cells over time; therefore avoid prolonged loading or delayed analysis.
7. Without washing, place the dish under a microscope suitable for live cell imaging. Important: fluorescence indicators are highly sensitive to light, therefore, avoid unnecessary light exposure.
8. Acquire images without changing the acquisition parameters to ensure comparability between different images.

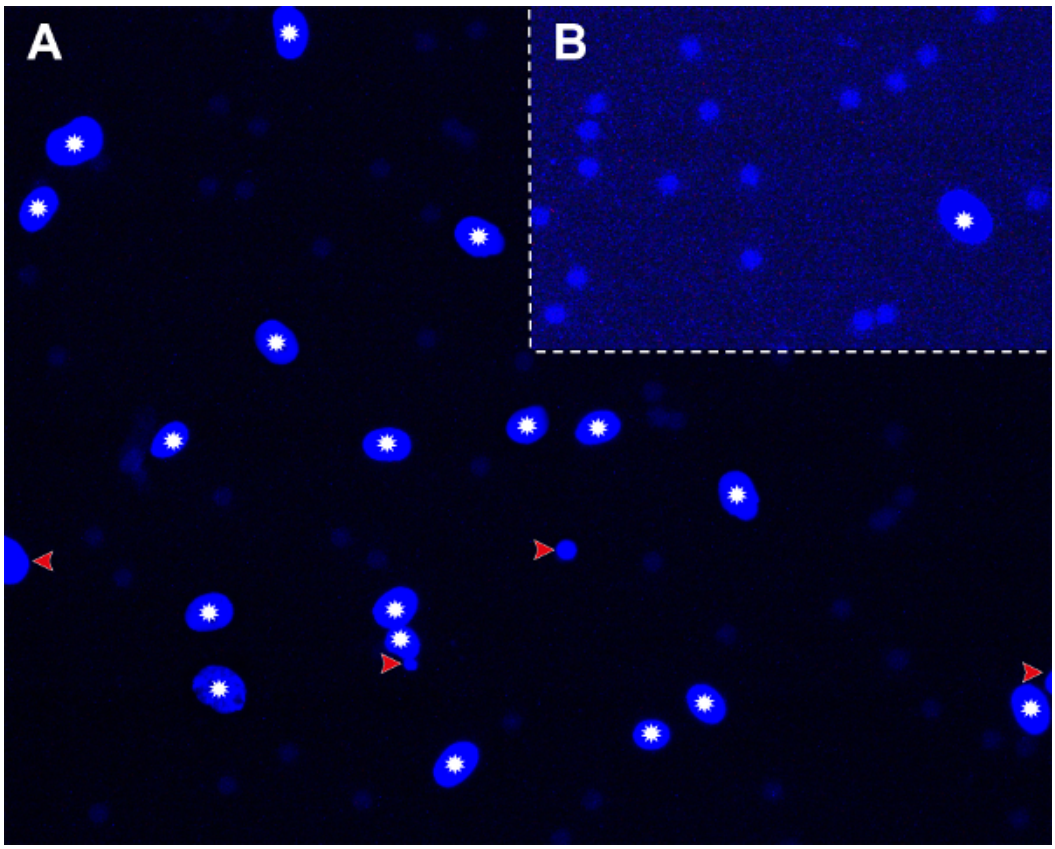
Representative Results

Dermal exposure to alkylating agents provokes erythema, blister formation and dermal ulceration that is associated with a wound healing disorder. Wound healing requires angio- and vasculogenesis which are based on migration of endothelial cells. Quantitative migration can be assessed by use of the Boyden chamber assay. As shown in **Figure 1C** exposure of EEC to the alkylating agent chlorambucil resulted in a significant decrease in cell migration[9]. Addition of the ROS-scavenger alpha linolenic acid (ALA) directly after exposure to chlorambucil for 24 hr significantly rescued this phenotype, almost to control levels.

The Boyden chamber assay is a suitable tool to assess the number (quantity) of migrated cells. However, the assay does not provide information about the migration behavior of the cells. **Figure 2** demonstrates that unexposed EEC are able to close the gap in the wound and healing assay within 24 hr[9]. In contrast, EEC exposed to the alkylating agent chlorambucil were unable to close the gap in the wound healing assay[9]. Moreover, cell tracking of single EEC revealed that under the influence of chlorambucil directed movement was not observed[9]. Treatment of EEC with ROS-scavengers was able to restore directed cell migration[9].

Mitochondrial ROS formation has been associated with impairment of cell migration[12]. As shown in **Figure 3** exposure of EEC to chlorambucil resulted in the breakdown of mitochondrial membrane potential[9]. Treatment of EEC with ROS-scavenger prevented mitochondrial damage and maintained the mitochondrial membrane potential.

In summary, all methods described in the protocol section are suitable tools to assess cell migration. Findings on the loss or preservation of the mitochondrial membrane potential may provide valuable hints regarding the underlying molecular mechanisms of impaired cell migration.



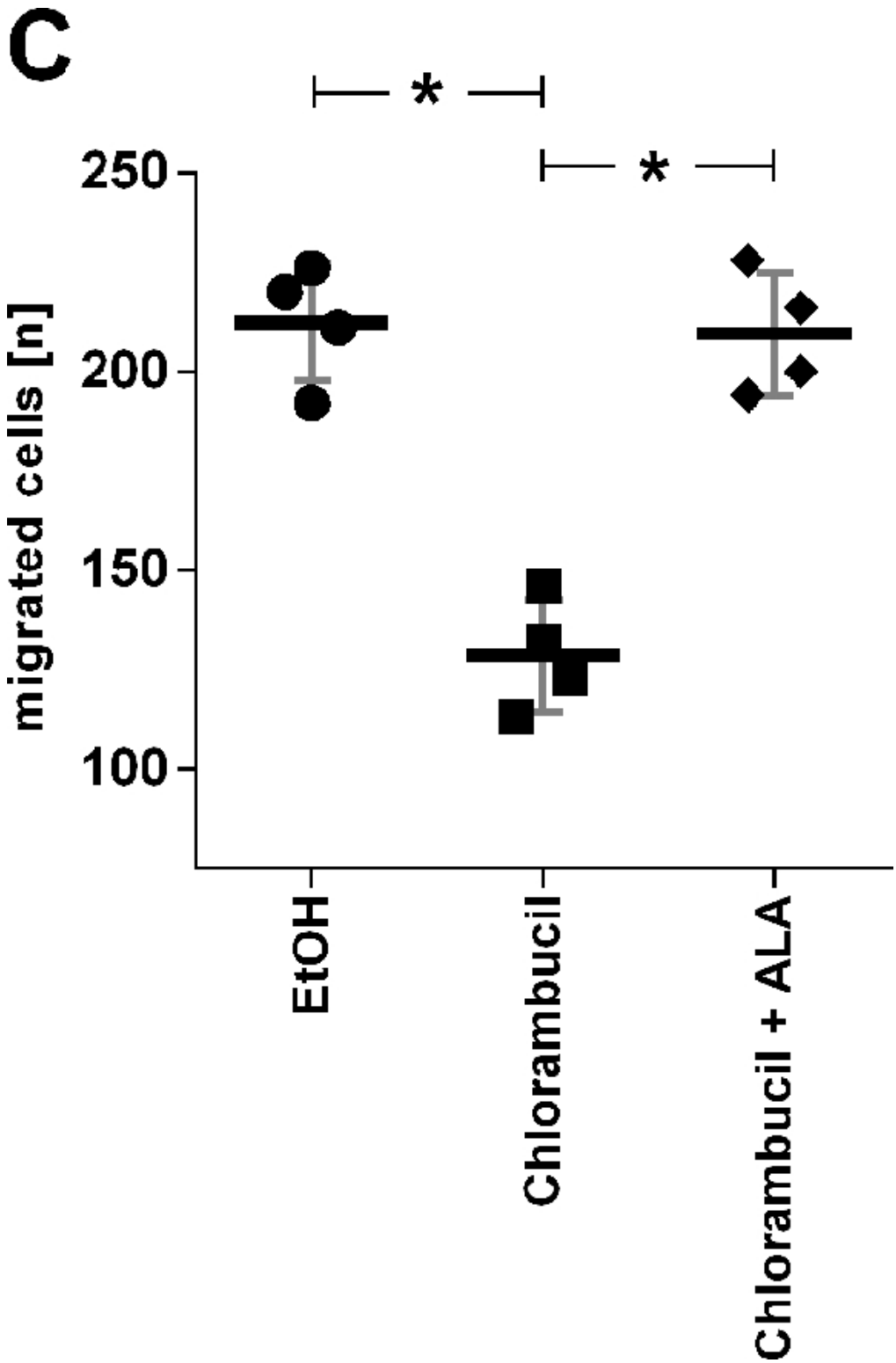
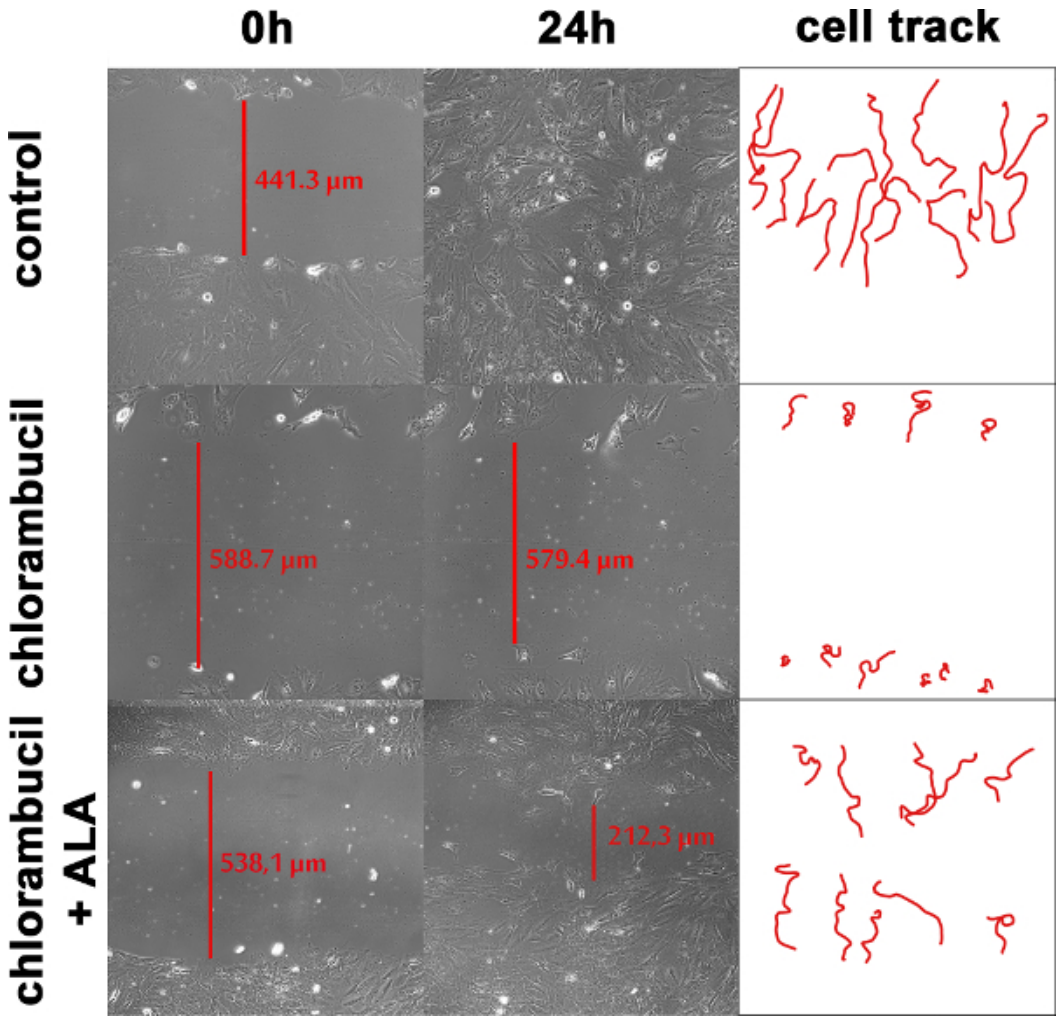


Figure 1. Boyden chamber assay. (**A**) EEC were seeded on Boyden chamber filter inserts, incubated for 8 hr, fixed and stained with DAPI. Asterisks indicate migrated EEC. In the presented image a total of 19 cells can be counted (**Figure 1B**). Red arrow heads indicate cells that have not migrated across the insert completely or that are not completely in the field of view and thus were excluded from cell counting. (**B**) The pores (8 µm) in the membrane become visible when using long exposure times for image acquisition. Do not confuse them with migrated cells. (**C**) Under control conditions an average of 212 cells migrated through the membrane. Chlorambucil exposure resulted in only 128 migrated cells. Treatment of chlorambucil-exposed EEC with the ROS-scavenger alpha linolenic acid (ALA) significantly rescued cell migration . Symbols represent individual results from one filter insert. Black horizontal bars indicate means (n = 4), error bars indicate SD and asterisks indicate significant differences. Please click here to view a larger version of this figure.


0h
24h
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control
chlorambucil
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441.3 µm
588.7 µm
579.4 µm
538,1 µm
212,3 µm

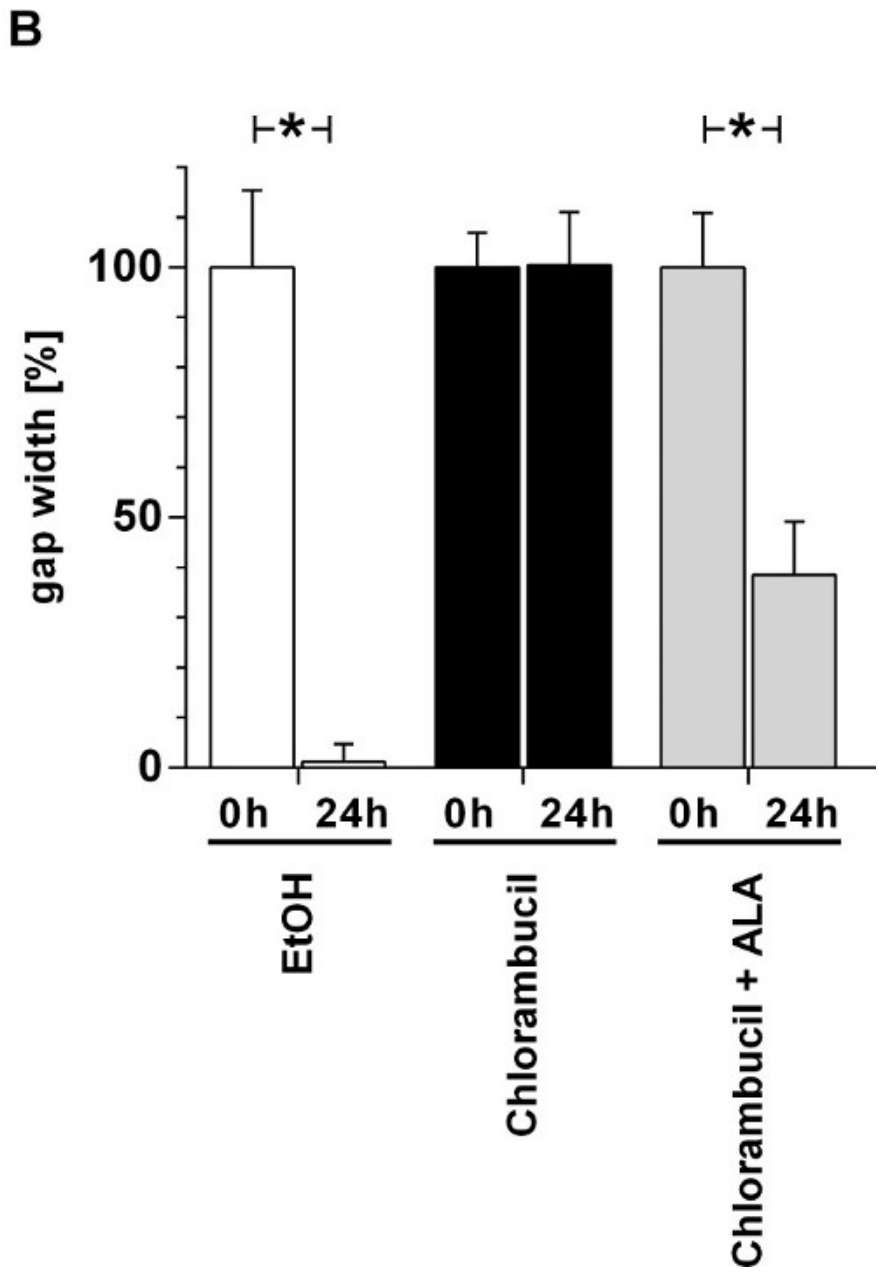

Figure 2. Wound healing assay/cell tracking. EEC were sham-treated (exchange of medium) or exposed to 12.5 µg/ml chlorambucil. After scratching, cells were investigated for 24 hr. (**A**) EEC were able to close the gap in the wound healing assay within 24 hrs whereas chlorambucil exposed EEC were unable to close the gap. Treatment with ALA resulted in a significant improvement of cell migration. (**B**) Mean values from 3 independent experiments each with 5 technical replicates per group (n = 15 per group) are shown. Error bars represent standard deviations and asterisks indicate statistical significant differences (One-way ANOVA, $p < 0.05$). Modified from Steinritz *et al.* (2014)[9]. Please click here to view a larger version of this figure.

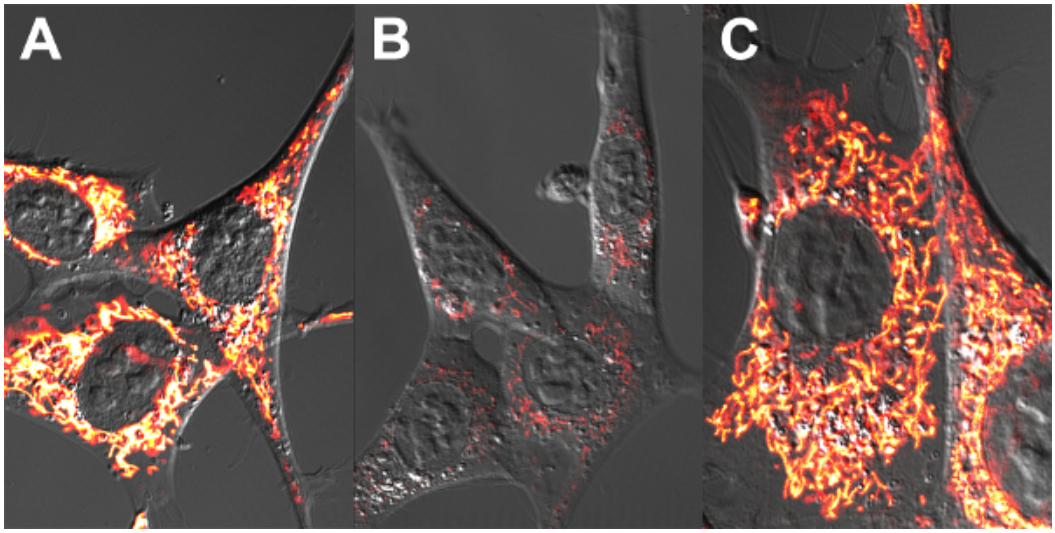

Figure 3. Assessment of the mitochondrial membrane potential. EEC were sham-treated, exposed to chlorambucil (12.5 µg/ml) or chlorambucil exposed and ALA (15 ng/ml) treated. 24 hr after exposure, cells were loaded with TMRM for 15 min and immediately imaged. (**A**) Unexposed EEC revealed a distinct signal after TMRM labeling indicating an unimpaired mitochondrial membrane potential. (**B**) Chlorambucil exposure resulted in a significant decrease of the TMRM signal. (**C**) Treatment of chlorambucil-exposed EEC with the ROS-scavenger alpha linolenic acid restored the mitochondrial membrane potential. Please click here to view a larger version of this figure.

Discussion

Dermal exposure to toxic chemicals often results in severe wound healing disorder. The underlying mechanisms are largely unknown. Wound healing is a complex process that consists of different phases (hemostasis, inflammation, proliferation and remodeling). Cell migration is involved in every phase, however, it is of utmost importance for the formation of the granulation tissue. Here, new blood vessels are formed either by angio- or vasculogenesis.

Both processes require unaffected migration of precursor and early endothelial cells. Assessment of cell migration and clarifying the underlying mechanisms for disturbed cell migration is highly relevant in order to develop new therapeutic strategies against toxicant induced wound healing disorders.

Cell migration can be assessed by different approaches. A commonly used technique is the Boyden chamber assay which was originally introduced to analyze antibody-antigen caused chemotaxis on leukocytes[13]. This assay is a quantitative endpoint assay that reflects the invasion capacity of cells from the upper compartment of a transwell insert to the lower compartment in response to a chemoattractant. In the technique described herein, no chemoattractant was added to the lower chamber. Thus chemokinesis (random migration without a specific chemoattractant) of EEC under control condition or in response to chlorambucil was investigated.

The insert consists of a porous membrane that allows three dimensional cell migration through the pores of the membrane. The pore size of the membrane has to be chosen in relation to the cell size: pores which are too small will prevent cell migration completely, pores which are too large will allow unhampered passing of all cells. For early endothelial cells, a pore size of 8 µm is recommended. The use of an inappropriate pore size will result in irreproducible results. In order to adapt the protocol to cells with different properties as EEC, we recommend starting with a pore size larger than the nuclei and increase the pore size step-by-step if necessary. For example, for small cells like leukocytes a pore size of 3 µm is recommended.

A critical issue is the determination of the incubation time until analysis. Over time, cell proliferation (at least in control groups) will take place. This in turn will amplify the number of cells that can penetrate through the membrane, potentially reducing the sensitivity of the method: even with slightly impaired migratory abilities, cells may be able to migrate over long periods of time. Therefore, long periods (*e.g.* 24 hr) are not recommended for endothelial cells. On the other hand, if the incubation is too short after cell seeding, only minor amounts of cells will have passed the membrane. An 8 hr incubation was found optimal. After the defined incubation time of 8 hr, cells are fixed, stained and manually counted.

There are three possible approaches for analysis: (1) Cells on top of the membrane (that have not migrated) are removed by a swab. Then the cells on the bottom side of the membrane (migrated cells) are stained either with a cytological (*e.g.,* hematoxylin) or a fluorescent dye (*e.g.,* DAPI) and are counted. (2) Alternatively, cells on the bottom side can be stained with a fluorescent dye first and are detached afterwards (*e.g.,* by the use of trypsin). Fluorescence is then quantified by the use of a fluorescence reader. (3) We recommend using dark colored porous membranes that block light transmission thereby allowing differentiation of non-migrated cells (cells on top of the membrane) and migrated cells (on the bottom of the membrane). After fluorescence staining (cytological dyes cannot be used) cells on the bottom side are counted using a fluorescence microscope. It is important not to confound the membrane orientation. Using an opaque membrane obviates the need to remove non-migrated cells. The use of opaque membranes, fluorescent staining and manual cell counting has turned out superior to the other approaches.

In general, the Boyden chamber assay is an assay that is easy to handle and thus it is a frequently used assay for the assessment of cell migration. However, there are two major disadvantages: (1) The Boyden chamber assay is an endpoint assay. That means, if the time point of

analysis is incorrect, the result may be misleading. (2) The assay is not easy to automate. Nevertheless, this assay is one of the most valuable tools for assessment of cell migration.

In contrast to the static Boyden chamber assay, the wound healing assay (also referred to as gap closure assay or scratch assay) is a dynamic assay in real time. It is a two dimensional migration assay without three dimensional cell invasion movements. After "wounding" of cell layers with a pipette tip, cells migrate into the gap. The assay is simple to perform and avoids the problems of a static endpoint assay. However, the assay has some challenges: (1) a live cell imaging microscopic system (37 °C, 5% CO_2 with humidified atmosphere) is required. Make sure that the cell culture dishes do not dry out. (2) Time-lapse image acquisition will result in large data files depending on the parameters (resolution, interval) used. (3) Scratching the cell layer with a pipette tip is not as easy as it may initially appear. Too much pressure will damage the dish surface which may result in artifacts. Moreover, the gap width may differ within the scratch and may differ between different investigators. An experienced investigator is recommended. (4) The number of samples that can be run in parallel is limited by the equipment. Nevertheless, the wound healing assay is another valuable tool for the quantitative and qualitative assessment of cell migration. Information about migration velocity, migration distance, directed migration can be gathered in one experimental setup. Cell tracking in a two dimensional system is not very difficult and can be performed with open source software (*e.g.,* ImageJ and MTrackJ).

Coating of the cell culture dishes is not only important for cell attachment, but has a significant influence on cellular function with regard to migration. Endothelial cells are routinely cultured on 0.1% gelatin-coated surfaces. Use of non-coated surfaced for endothelial cell cultivation resulted in decreased proliferation[14]. Other coatings (*e.g.,* fibronectin) were found also suitable for short-term cultivation, however, gelatin coatings showed superior performance in long-term cultivation experiments[15].

There are numerous fluorescence dyes on the market for live cell imaging. We have chosen TMRM to investigate the mitochondrial membrane potential. Many other dyes (*e.g.,* JC-1, TMRE) exhibit comparable performance. In contrast to monochromatic probes (TMRM, TMRE), JC-1 fluorescence shifts from green to red after accumulation in the mitochondria. This allows a ratiometric semi-quantitative analysis of the mitochondrial polarization state but hampers dual staining experiments (*e.g.,* using tracker dyes to identify mitochondria)[16]. In our experience the dye itself is less critical for the experiment whereas the experimental design, cell handling and acquisition is challenging and requires much more attention. Almost all fluorescent dyes are light sensitive. Therefore, cell loading should be done in the dark. Cell loading requires 15-30 min. Prolonged loading time does not result in increased signals and is not recommended. Although specific signals could be detected after long loading periods, the dye might accumulate in subcellular compartments or might even be discharged from the cell. As a consequence, cells should be loaded just prior to analysis and not necessarily at the beginning of the experiment. The dye loading concentration usually is in the range of 10 µM. However, with TMRM much lower concentrations (20 nM) were found sufficient for labeling and still gave reasonable results. As shown in **Figure 3** labeling 24 hr post chlorambucil exposure resulted in a distinct loss of mitochondrial membrane potential. Treatment of chlorambucil exposed EEC with ROS-scavengers was able to maintain mitochondrial membrane potential at 24 hr after the initial exposure.

In summary, Boyden chamber assay, wound healing assay in combination with single cell track analysis are valuable tools to assess migration behavior with regard to qualitative and quantitative aspects. Labeling of mitochondrial potential with TMRM can help to identify underlying mechanisms.

Disclosures

The authors have nothing to disclose.

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