Interaction of primary human trabecular meshwork cells with metal alloy candidates for microinvasive glaucoma surgery

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Abstract

Background: Microinvasive glaucoma surgery (MIGS) is a relatively new addition to the glaucoma treatment paradigm. Small metallic stents are inserted into the trabecular meshwork in order to increase aqueous humour drainage. MIGS procedures are rapidly being adopted owing to a more favourable side effect profile when compared with traditional surgery. Remarkably, this rapid rate of utilization has occurred without any published studies on the effect of metal alloys used in these stents on human trabecular meshwork cells (HTMCs). Therefore, this study aimed to determine the effect of candidate metal alloys for MIGS on HTMC morphology, viability and function.

Methods: Human trabecular meshwork cells were cultured on the surfaces of titanium (polished and sandblasted), a titanium–nickel (nitinol) alloy and glass (as control substratum). Fluorescence imaging was used to assess cell morphology and spreading. A lactate dehydrogenase cytotoxicity assay, cell death detection ELISA, MTT cell viability assay, BrdU cell proliferation assay and fibronectin ELISA were also conducted.

Results: Cells cultured on sandblasted titanium exhibited significantly greater spreading than cells cultured on other substrata. In comparison, HTMCs cultured on nitinol displayed poor spreading. Significantly more cell death, by both necrosis and apoptosis, occurred on nitinol than on titanium and glass. Also, cell viability and proliferation were suppressed on nitinol compared with titanium or glass. Finally, HTMCs on both titanium and nitinol produced greater amounts of fibronectin than cells grown on glass.

Conclusions: Substratum topography and metal alloy composition were found to impact morphology, viability and function of primary HTMC cultures.

Key words: alloy, glaucoma, stent, trabecular meshwork.

Introduction

The traditional treatment paradigm for primary open-angle glaucoma recommends that patients start with the daily administration of topical eye drops followed by laser therapy to lower intraocular pressure (IOP). In more recent years, some practitioners have adopted laser trabeculoplasty earlier in the paradigm-often using medical and laser therapy in conjunction. Typically, it is only when maximally tolerated medical and/or laser therapy fails that the
paradigm recommends advancement to surgical interventions. As with any treatment plan that relies heavily on chronic medical therapy, high rates of non-compliance, side effects and cost impede sustained treatment success. Low compliance significantly increases the risk for disease progression. Therefore, the use of surgery at an earlier stage of treatment has always been an enticing option.

Thus, it is no surprise that the recent introduction of microinvasive glaucoma surgery (MIGS) has shaken the current treatment paradigm of glaucoma. A novel category of MIGS involves the insertion of stent devices into the trabecular meshwork through a minimally invasive internal (ab interno) approach with the goal of augmenting aqueous humour outflow and hence lowering IOP. The first MIGS device to gain widespread use was the iStent (Glaukos Corporation, Laguna Hills, CA, USA). It is a 1-mm titanium device that is surgically implanted into the trabecular meshwork to aid in aqueous humour drainage. In cultured human anterior segments, the iStent has been shown to reduce IOP to within normal range. A second MIGS device called the Hydrus Microstent (Ivantis Inc., Irvine, CA, USA) is constructed of a titanium–nickel (nitinol) alloy and so far has displayed promising clinical efficacy. A major advantage of MIGS is that efficacy does not depend on patient compliance and the treatment is low risk with a very low side effect profile compared with traditional surgery. Some have predicted that microinvasive procedures will eventually become first-line treatment for glaucoma and pose fewer long-term costs for the health-care system.

Although practitioners are enthusiastic towards the rapid adoption of MIGS into clinical practice, major unresolved issues include lack of knowledge on the effects of product materials on surrounding cell structures, their safety and biocompatibility. Such issues could affect both the structure and function of the tissues leading to unpredicted long-term complications. To our knowledge, no studies exist describing the morphological or functional effects of stent biomaterials on human trabecular meshwork cells (HTMCs). Research in other fields has shown that various types of biomaterials, including those used in MIGS stents, can influence both the morphology and function of cells. For example, cardiovascular stents made of titanium alloys have been shown to influence endothelial cells, which express extracellular matrix proteins similar to HTMCs. In addition, dental literature has suggested that nitinol alloys induce greater oxidative stress and decrease the viability of oral mucosa cells compared with pure titanium. The purpose of this study was to investigate the effect of candidate metal alloys for ab interno microstenting on the morphology, viability and function of HTMCs. Our aim was to obtain insights on the biocompatibility of these materials with trabecular meshwork cells.

**Methods**

**Metal alloy preparation**

The three metal alloys used were machine-polished grade 2 (commercially pure) titanium (Titanium Metal Suppliers, Poway, CA, USA), machine-polished titanium–nickel (50%) alloy (American Elements, Los Angeles, CA) and sandblasted and acid-etched pure titanium (Institute Straumann, Burlington, ON, Canada). Grade 2 titanium and nitinol rods were cut into discs (15 mm diameter, 1 mm thick). Machine-polished titanium (Fig. 1a) and nitinol surfaces were prepared by polishing under water using emery papers of different grit sizes for 1 h to generate uniform surface topographies. Sandblasted and acid-etched titanium surfaces were prepared by the manufacturer (Fig. 1b). The surface topography of grade 2 titanium and nitinol samples was evaluated using three measurements from a Surftest SJ-210 mechanical stylus profilometer (Mitutoyo, Kanagawa, Japan) to establish that the surface roughness of each sample was within 0.1 μm Ra. In addition to the three metal substrata, glass coverslips (12 mm, #1 thickness, Fisher Scientific Company, Ottawa, ON, Canada) were used as control surfaces. All four substrata were cleaned and sterilized in low-temperature, radio-frequency glow discharge argon plasma using a PDC-32G plasma cleaner (Harrick Plasma, Ithaca, NY, USA) for 4 min.

**Cell culture**

Primary HTMCs were purchased from ScienCell Research Laboratories, Carlsbad, California, USA. The cells were isolated from the juxtaocular and corneoscleral regions of the human eye and previously examined by immunofluorescence using well-characterized antibodies to proteins (α-smooth muscle actin and fibronectin) considered to be TM markers. Three to four primary HTMC lines were thawed from storage in liquid nitrogen and propagated in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution. All cell culture reagents were purchased from Invitrogen (Gibco-BRL, Burlington, ON, Canada) unless otherwise stated. Cells were incubated at 37°C and 5% CO₂ until the cells were 80–100% confluent. Substrata were placed in wells of a 24-well culture plate. Subsequently, HTMCs were seeded at 1.0 × 10⁵ cells/well. For each donor, two samples of each metal and control substrate were tested. The cells were incubated for

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24 h at 37°C and 5% CO₂. Experiments were performed on first-passage to third-passage HTMCs.

**Fluorescence microscopy**

After 24 h of incubation, the HTMCs were fixed, stained and imaged using procedures successfully employed in our previous studies.²⁰ Following fixation and permeabilization, the samples were washed with phosphate-buffered saline (PBS) and treated with 1% bovine albumin (BSA) for 40 min at room temperature to block non-specific binding. Samples were then incubated with primary antibody to vinculin (Sigma-Aldrich, Oakville, ON, Canada) at 1:100 dilution in 1% BSA in PBS for 1 h. This was followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody for 1.5 h (1:200 dilution in 1% BSA in PBS, Life Technologies Inc., Burlington, ON, Canada) to reveal vinculin, which is enriched in focal adhesions. Filamentous actin was stained using rhodamine-conjugated phalloidin (1:100 dilution in 3% PBS, Cytoskeleton Inc., Burlington, ON, Canada). Nuclei were counterstained using Vectashield Mounting Medium (Vector Labs, Burlington, ON, Canada) containing 4',6-diamidino-2-phenylindole. The cells were imaged using a Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss Canada, Toronto, ON, Canada) equipped with an AxioCam MR3 and AxioImager software (Carl Zeiss Canada).

**Quantification of HTMC attachment and spreading**

Cell attachment was quantified by counting the 4',6-diamidino-2-phenylindole-stained nuclei from 10 randomly selected and non-overlapping fields of view from samples of each donor. Spreading was quantified by measuring the planar area of 10 cells from each of the three donors using ImageMaster 5.0 software (HORIBA Scientific-Photon Technology International, Kyoto, Japan).

**Colorimetric assays**

After 24 h of incubation on the substrata, five colorimetric assays were performed including a lactate dehydrogenase (LDH) assay (Sigma-Aldrich), a cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche, Laval, QC, Canada), a human fibronectin ELISA (Abcam, Cambridge, MA, USA), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) assay (Sigma-Aldrich) and 5-bromo-2’-deoxyuridine (BrdU) proliferation assay (Cell Biolabs Inc., Burlington, ON, Canada) as per the manufacturers’ instructions. Absorbance was determined using a spectrophotometer microplate reader (Bio-Rad, Mississauga, ON, Canada) at wavelengths specified by the manufacturers’ instructions. The LDH and cell death detection ELISA provided measures of cytotoxicity by evaluating the amount of cell death by necrosis and apoptosis pathways, respectively. The fibronectin ELISA estimated the relative amounts of fibronectin secreted. The MTT assay assessed cell viability by comparing metabolism in experimental cells with control cells. The BrdU proliferation assay estimated cell proliferation by detecting the amount of 5-bromo-2’-deoxyuridine incorporated into cellular DNA during cell proliferation. In the LDH assay and cell death detection ELISA, total protein concentration in each sample was measured (Bio-Rad Protein Assay, Mississauga, ON, Canada) to normalize the absorption data. For the fibronectin ELISA, cell number was determined by manual counting under the Nikon Eclipse TS100 inverted routine microscope to normalize the absorption data.
**Scanning electron microscopy**

Images were obtained using a Hitachi 3400-N variable pressure scanning electron microscope (Hitachi Ltd, Tokyo, Japan) at the Western University Biotron Experimental Climate Change Research Centre.

**Statistical analysis**

Data were analysed statistically using one-way ANOVA and Tukey’s multiple comparisons test (GraphPad Software, La Jolla, CA, USA). Differences were accepted as statistically significant at $P < 0.05$. All data are expressed as means ± SD.

**RESULTS**

**Cell morphology**

Fluorescence microscopy of HTMCs revealed distinct differences in cell morphology depending on the substratum upon which the cells were cultured. HTMCs cultured on glass, machine-polished titanium and machine-polished nitinol are compared in Figure 2a–c. The HTMCs on glass (Fig. 2a) were stellate-shaped, had many focal adhesions and were randomly oriented (unaligned) on the substratum. In comparison, the HTMCs on machine-polished titanium (Fig. 2b) appeared spindle-shaped, displayed few focal adhesions and were aligned in a pattern coinciding with the grooves on the metal surface. Lastly, the HTMCs grown on machine-polished nitinol (Fig. 2c) exhibited minimal cell spreading, had very few focal adhesions and were randomly oriented.

Human trabecular meshwork cells cultured on machine-polished titanium were compared with cells cultured on sandblasted and acid-etched titanium in Figure 3. As described earlier, HTMCs on machine-polished titanium (Fig. 3a) were spindle-shaped, with few focal adhesions and an aligned distribution. In contrast, HTMCs on sandblasted and acid-etched titanium (Fig. 3b) were extensively spread and stellate-shaped with many focal adhesions and were randomly oriented on the substratum. In summary, culturing HTMCs on different substrata and surface topographies led to distinct cell morphologies.

**Cell necrosis and apoptosis**

Cell necrosis and apoptosis were measured for HTMCs grown on various substrata using the LDH assay and cell death detection ELISA, respectively. Compared with glass control, no significant changes in cell necrosis ($P > 0.05$, Fig. 4a) nor apoptosis ($P > 0.05$, Fig. 4b) were observed in HTMCs after 24 h culture on polished titanium. In contrast, significantly more cell necrosis ($P = 0.013$, Fig. 4a) and apoptosis ($P = 0.012$, Fig. 4b) were observed on nitinol than on titanium or glass.

**HTMC function, proliferation, and viability**

Human trabecular meshwork cell fibronectin production after 24 h was measured using the fibronectin ELISA. Significantly more fibronectin production was observed on polished titanium and nitinol than on glass ($n = 4$, $P = 0.0033$, Fig. 4c). There was no significant difference in fibronectin production when comparing HTMCs grown on titanium and nitinol ($P > 0.05$, Fig. 4c). Significantly less proliferation was found on nitinol compared with other substrata as shown by the BrdU assay ($n = 3$, $P = 0.01$, Fig. 4d).

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**Figure 2.** Effects of different materials on the morphology of human trabecular meshwork cells (HTMCs). HTMCs were labelled for nuclei (blue), filamentous actin (red) and vinculin (green) and viewed under 20× magnification. The HTMCs on glass (a) had many focal adhesions, a random growth pattern and a stellate shape. The HTMCs on machine-polished titanium (b) appeared spindle-shaped, had few focal adhesions and a growth pattern that coincided with the grooves on the metal surface. The HTMCs grown on the machine-polished nitinol (c) exhibited minimal cell spreading, a random growth pattern and very few focal adhesions. At least 10 different fields of view per sample were acquired. One representative field is shown for each sample. Scale bars are 20 μm.
whereas there was no significant difference between proliferation on titanium and glass \((P > 0.05, \text{Fig. 4d})\). Consistent with results of the necrosis, apoptosis and proliferation assays, significantly lower cell viability was observed on nitinol compared with other substrata as shown by the MTT assay \((n = 3, P = 0.019, \text{Fig. 4e})\).

### Quantification of the effects of substratum on cell attachment and spreading

Cell attachment and spreading were quantified from the fluorescence microscopy images obtained with AxioImager software using ImageMaster5. There was no significant difference \((P > 0.1, \text{Fig. 5a})\) in...
the number of cells adhered to each of the substrata. However, there was significantly greater cell spreading on the sandblasted and acid-etched titanium than on other substrata \( (n = 3, P = 0.012, \text{Fig}. \text{5b}) \) and significantly less spreading on the nitinol surface than on glass \( (n = 3, P = 0.017, \text{Fig}. \text{5b}) \).

**DISCUSSION**

Materials currently used in the construction of microstent devices include titanium and nitinol alloys. Limitations on using alloys include the release of toxic particles through corrosion and wear processes that may result in reduced biocompatibility. Limitations on using alloys include the release of toxic particles through corrosion and wear processes that may result in reduced biocompatibility. Biocompatibility may be assessed using factors such as cellular morphology, viability, proliferation and function. This *in vitro* study demonstrated that both metal alloy composition and surface texture can impact the morphology, viability, proliferation and function of HTMCs in as little as 24 h after contact. To our knowledge, this is the first study of the effects of metal alloys on primary HTMCs. The time point of 24 h was chosen because titanium and nitinol alloys affect cell behaviour *in vitro* at intervals ranging from minutes to days. In addition, nitinol discs immersed in cell culture media have been shown to release the most amount of nickel after 1 day, with the amount steadily declining by 5 days. Finally, previous studies using cultured trabecular meshwork cells have established that 24 h is an ideal time point for experimental evaluation in order to avoid confounding factors such as excessive cell confluence and apoptosis.

Titanium and nitinol are widely used biomaterials in the production of cardiovascular stents, dental appliances and orthopaedic implants. Nitinol is an attractive alloy for biomedical applications because of its desirable combination of qualities such as super-elasticity, strength and shape memory. The concern with nitinol is its high nickel content (55%) and the conflicting evidence on its corrosion resistance and biocompatibility. Nitinol has been found to induce oxidative stress in various tissue types. For example, in an *in vitro* study using mouse fibroblasts, the concentration of the oxidative stress marker 8-hydroxy-20-deoxyguanosine was measured after cells were exposed to six types of orthodontic archwires for 48 h. All nitinol containing archwires were found to induce oxidative stress. When HTMCs are exposed to oxidative stress in culture, a morphological response involving alteration of the actin cytoskeleton is observed along with an increase in cell death. In addition, nickel has been observed to suppress alpha actin expression in rat vascular smooth muscle cells. One factor strongly expressed in HTMC cultures and necessary for HTMC spreading is alpha smooth muscle actin. Materials that disrupt the HTMC actin cytoskeleton can cause loss of contact with neighbouring cells, hinder cell proliferation and lead to destabilized trabecular meshwork architecture. Results from this present study showed that HTMCs cultured on nitinol exhibited very minimal cell spreading and had very few focal adhesions. This is consistent with the hypothesis that nitinol may induce oxidative stress and disrupt actin network formation. Without an intact cellular actin network, tissue integrity would be lost *in vivo* owing to the constant stress trabecular meshwork cells are subjected to in their environment. Consistent with this idea, our cytotoxicity assays revealed that nitinol surfaces induce higher levels of apoptosis and necrosis than glass. It is well documented that oxidative stress from nitinol corrosion products can produce DNA damage, apoptosis and necrosis and lead to adverse physiological effects in human peri-implant cells. Mechanisms range from cell toxicity and mutagenesis to the release of reactive oxygen species. Additional studies are required to determine if the
minimal cell spreading and increased cell death observed in HTMCs on nitinol are due to an oxidative stress response. The HTMCs used in the present study were derived from healthy human subjects without glaucoma. It is possible that HTMCs from glaucomatous patients may differ in their sensitivity to insults such as oxidative stress from nitinol.

The relatively low cellular proliferation and viability exhibited by HTMCs grown on nitinol are similar to what has been observed in vascular cells in vitro. Similar to HTMCs, decreased proliferation has been observed in endothelial cells cultured on nitinol compared with glass control. Furthermore, another study by Shih et al. found that nitinol corrosion products inhibit rat aortic smooth muscle cell proliferation. The low proliferation rate of HTMCs on nitinol may be due to a similar process. Although low cellular proliferation rates may be disruptive to trabecular meshwork anatomy, it should be pointed out that minimal cell spreading and local reductions in cell viability may actually be desirable responses to an implantable device. This is based on the fact that one of the most common postoperative complications following MIGS is stent obstruction. Owing to the small size of these devices, exuberant cell proliferation may cause blockage. In addition, there is evidence that surface morphology and finish can impact the amount of corrosion products released by nickel alloys, and further investigation into the type of surface that will minimize ion release will be essential towards popularizing nitinol as a biomaterial. As the Hydrus Microstent has a fine surface coating of pure titanium, the potential corrosive effects of nitinol would unlikely be a problem unless the device was altered in some way at the time of surgical implantation. Attention to surgical technique and the consequences of excessive manipulation are likely important elements in device performance.

Titanium is widely implemented in biomaterials owing to its excellent characteristics such as high resistance to corrosion, high specific strength and greater biocompatibility compared with other metal alloys. Titanium alloys have been assessed in various studies across specialties and have been shown to be non-cytotoxic and relatively inert both in vitro and in vivo. This is attributed to the presence of an oxide layer that forms on titanium surfaces when immersed in oxidizing environments such as cell culture media. However, evidence exists to suggest titanium may impact cellular proliferation, function and morphology. For example, cardiovascular stents made of titanium alloys have been shown to alter the morphology and function of endothelial cells, which express extracellular proteins similar to HTMCs. In addition, specific types of titanium alloys have been shown to inhibit the normal differentiation of bone marrow stromal cells in vitro. It is useful to note that in the aforementioned studies as well as the present study, grade 2 commercially pure titanium was utilized. Some stents are made using medical grade (grade 5) titanium alloy, which is stronger than commercially pure titanium and even less prone to corrosion. In the present study, HTMCs displayed robust attachment to titanium surfaces. Quantitative analyses assessing cell apoptosis, necrosis, proliferation and viability were in agreement with our morphological findings. Collectively, our results are consistent with numerous studies indicating that titanium is a biocompatible substrate for cell culture and clinical use. In addition, titanium-based MIGS stents such as the iStent possess a thin coating of heparin that may modulate the initial interaction of cells with the stent.

Varying the surface topography of metal alloys can influence the growth, adhesion and morphology of various cell types. Roughened titanium surfaces aid in the adhesion of osteogenic cells and the mechanical stability of osseous implants. Moreover, rat endothelial cells develop a more complex extracellular matrix and exhibit increased proliferation on titanium surfaces with rougher topography. Furthermore, Lu et al. observed that rougher titanium surfaces induce greater rat endothelial cell adhesion and migration. They also found that specific nano-patterned surfaces strongly enhanced endothelial cell function. Consistent with these findings, we observed marked cell spreading and focal adhesion formation on the sandblasted and acid-etched titanium surface. In contrast, less spreading and focal adhesion formation were found for HTMCs grown on polished titanium. These results suggest that HTMCs adhere more strongly to rougher surfaces. Interestingly, HTMCs on polished titanium were observed to grow along the grooves in the metal surface. Intravascular stents have taken advantage of this phenomenon by patterning stent surfaces to encourage endothelial cell growth along the stent in a direction that will not cause occlusion. Our findings suggest that HTMCs are also responsive to surface patterning, and perhaps, a surface texture with parallel grooves could be applied to MIGS devices to help prevent occlusion.

Overall, this study determined that the composition and surface texture of metal alloy candidates for glaucoma surgery impact HTMC morphology, viability and function. We explored the biocompatibility of two metals currently being utilized in the production of MIGS devices. Ultimately, our results highlight the need for further research. It should be noted that our results are not necessarily indicative of the surgical success of these metals in the complex in vivo environment. There are a number of limitations of using cultured cells for these studies. For example, cells in vivo exist in 3-D and interact with extracellular matrix...
and other cell types; whereas in vitro, cells are propagated in 2-D in predefined medium and on predefined substrates. Therefore, a number of cues arising from cell–matrix and cell–cell interactions are absent in vitro. In addition, many components of homeostatic in vivo regulation (nervous system, endocrine system and metabolic integration) are lacking in in vitro cultures. As such, in vivo studies evaluating the response of HTMCs and the surrounding matrix to implantable metal alloys would be a useful bridge between in vitro and clinical studies. For example, Saheb et al. recently published a histological study of the Hydrus stent implanted in an animal model for 16 weeks and found little inflammatory response and good biocompatibility. Although our study did not assess the inflammatory process, these histological findings are of interest. In addition, studies are ongoing to assess alternative implantable materials, as well as explore the effect of varying surface patterns on HTMC behaviour. With MIGS having a prominent role earlier in the glaucoma treatment paradigm, studies that examine safety and short-term and long-term efficacy at the cellular, tissue and organ level will be valuable in optimization of device performance and understanding of how MIGS best fits into this paradigm.

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References