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Mueller, P.P., May, T., Perz, A., Hauser, H., Peuster, M.
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(2006) Biomaterials, 27 (10), pp. 2193-2200

Control of smooth muscle cell proliferation by ferrous iron

Abbreviated title: Cellular response to iron stents

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Keywords: Biocompatibility; Biodegradation; Cell proliferation; Corrosion product; Degradation; Intravascular stent; Intimal hyperplasia

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Abstract

This study was conducted to determine the interaction of individual corrosion products from biodegradable iron stents with cells from the adjacent tissue. The response of human umbilical venous smooth muscle cells to an excess of ferrous ions was investigated in a cell culture model at the phenotypic and at the molecular level. When soluble ferrous ions were added to the cell culture medium the cell growth rate was reduced. Gene expression profiling indicated a reduction in the amounts of mRNA from genes that are required for cell proliferation. In addition, mRNA was regulated from multiple genes involved in iron homeostasis, DNA replication and lipid metabolism. In conclusion, ions released from iron stents could reduce the vascular smooth muscle cell proliferation rate by influencing growth-related gene expression and may therefore play a beneficial role in antagonizing restenosis in vivo.

1. Introduction

1. Introduction

Current cardiovascular implants have until recently been considered to be stable. However, there is increasing evidence of ion leakage from metallic implant materials. The accumulation of implant degradation products in the surrounding tissue has been demonstrated for orthodontic implants, orthopedic implants, and also for cardiovascular implants [1-8]. Even implants made of stainless steel slowly corrode under physiological conditions [6]. Tungsten was the first cardiovascular implant material used that showed complete dissolution in humans [9]. It was demonstrated that corrosion of the coil was the cause of degradation [10].

The conception of biodegradable cardiovascular implants based on the metal corrosion has been introduced only recently [11]. Degradable stents are of particular interest for pediatric applications due to the fact that the vessels are growing. In relation to the vessel, the stent becomes too narrow and may restrict the blood flow. This complication could be avoided by using a biodegradable stent that would disintegrate in time. In addition, in stent restenosis due to excessive neointimal smooth muscle cell (SMC) proliferation is a frequent problem. As a proof of concept, both, safety and suitability of stents made of pure iron were demonstrated. Iron stents implanted in the descending aorta of rabbits did not cause any pronounced neointimal hyperplasia [11]. The concept was subsequently applied to magnesium-based coronary [12] and peripheral [13] stents.

Iron is an essential co-factor for a multitude of enzymes involved in diverse physiological processes such as oxygen binding, DNA synthesis and redox enzyme activity. Uptake and intracellular iron accumulation are highly regulated processes that are controlled by intricate mechanisms at transcriptional, posttranscriptional and translational levels [14]. Nevertheless, excess iron can cause disease [15] and corrosion products from iron implants could affect the adjacent tissue.

Several observations argue against a significant cytotoxic effect of iron and its degradation products in vivo. The corrosion rate of elementary iron in vivo is slow and has a low toxicity [11]. The slow degradation rate and the small amount of iron in a stent (40 mg) in relation to the iron-load of whole

blood (400-500 mg/l) make any systemic toxicity extremely unlikely. During degradation, Fe(II) ions are first liberated from the surface of metallic iron. These ions could either be oxidized to Fe(III) or they could interact with nearby cells. To assess the response of cells to Fe(II) a cell culture model was established. Even though immortalized cell lines are easier to handle and give more consistent results, such cells differ considerably from primary cells, such that primary cells were considered to more closely represent to the in vivo situation [16]. With the possible exception of the initial wound healing phase, an iron stent will be surrounded mainly by SMCs during the largest part of its life time. Therefore, primary human vascular SMCs were considered to be highly relevant for these investigations. Fe(II) was added to the cell culture medium as Fe(II)-D-gluconate-dihydrate, an oral drug used to treat iron deficiency. The results show that high Fe(II) concentrations inhibit cell proliferation. Gene expression profiling was used to obtain an unbiased broad view of the molecular processes responsible for the reduced cell growth rate. Most importantly, a significant number of genes required for cell proliferation, cell cycle progression or DNA replication were down regulated in the presence of iron. The results suggest that ions released from iron stents could antagonize restenosis by reducing excessive vascular cell proliferation.

2. Materials and Methods

2.1. Isolation and cultivation of primary cells

Primary human vascular SMCs from umbilical cord veins were isolated by standard protocols [17]. To reduce the individual donor-dependend variation, primary cells from several donors were pooled. To allow the reproduction of the results aliquots of the cell culture were stored frozen in liquid nitrogen until use. After thawing, the cells were cultivated in SmGM (SmGM-2-Bulletkit Nr. CC-3182, Cambrex) at 37 °C in a 5 % CO₂ atmosphere. For cell cultivation in the presence of excess iron the cells were seeded at a density of 5×10^3 in 100 µl per well of a 96-well microtiter dish and incubated for 24 hrs under standard conditions. Fe(II)-D-gluconate-dihydrate was added (Aldrich, 344 427) at a concentration of 0 to 0.1 mg/ml. For the mRNA profiling experiments Fe (II)-gluconate-dihydrate was added to the culture medium at a concentration of 0.03 mg/ml.

2.2. Cell proliferation

Cell proliferation was determined based on the metabolic activity of mitochondrial dehydrogenases using a WST-1-Test [18] (Roche, Nr. 1644807). In short, after the cultivation the cell growth medium was removed and replaced by 100 µl of medium containing 10 % WST-1 reagent. After incubation for 2 hours at 37 °C in 5 % CO₂ the optical density at 440 nm was determined relative to the OD at 650 nm using an ELISA multiplate reader (Sunrise, Tecan). The background was adjusted using 100 µl medium with 10 % WST-1 reagent without cells.

2.3. DNA synthesis

BrdU-incorporation into DNA was determined as recommended by the manufacturer (BrdU incorporation kit Nr 1647229, Roche). Briefly, 10 µl BrdU labeling solution was added to 100µl of the cell culture medium. After incubation for 2 h under standard cell culture conditions, the labeling solution was removed and the cells were fixed at room temperature for 30 min with 200 µl FixDenat. The fixing solution was removed and replaced with anti-BrdU-POD working solution, followed by 60 min. incubation at ambient temperature. The cells were washed three times and 100 µl substrate solution was added to each well. After further 15 min incubation the solution had turned blue and 25 µl 10 % sulfuric acid was added, followed by 15 min. incubation at room temperature when the solution turned. After shaking for 1 min. the OD was determined at 450 nm and a reference wavelength of 690 nm in an ELISA multiplate reader (Sunrise, Tecan). As a background control 100 µl of cell culture medium was mixed with 10 µl BrdU labeling solution.

2.4. RNA isolation

SMCs derived from the veins of 12 umbilical cords were pooled and cultured in T-75 flasks in SmGM medium. Iron salts were added to the medium 24 hours after reseeding first passage cells and the incubation was continued for 12 hours and 24 hours, respectively. Then the medium was removed, cells were washed with PBS and detached by treatment with Accutase (PAA Linz, Austria). RNA was isolated from these cells and treated with DNase according to standard procedures (RNeasy Mini Kit Nr. 74104, Qiagen).

2.5. DNA Microarray Hybridization and Analysis.

RNA expression profiling was done as previously described [19]. Briefly, quality and integrity of the total RNA extracted from cultured cells was determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). Probes were synthesized as specified by the manufacturer (Affymetrix; Santa Clara, CA). Briefly, 3 µg of purified total cellular RNA was reverse transcribed using 100 pmol of a T7T23V primer (Eurogentec; Seraing, Belgium) containing a T7 promoter. The cDNA was then used in an in-vitro transcription reaction in the presence of biotinylated nucleotides. The concentration of biotin-labeled cRNA was determined by UV absorbance. 12.5 µg of each biotinylated cRNA sample was fragmented and added to a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre). Samples were hybridized for 16 hours to an identical lot of the Affymetrix GeneChip HG_U133A containing 22 283 different probe sets corresponding to 14239 independent Unigene clusters (Unigene build 155). After hybridization the GeneChips were washed, fluorescently stained with SA-PE (streptavidin-phycoerythrin) and scanned using an Affymetrix GeneChip fluidic station with a scanner.

2.6. Data Analysis

Analysis of microarray data was performed as previously described [19] with minor modifications. Briefly, genes were considered as regulated when their fold-change value is greater than or equal to 1.5 or less than or equal to -1.5. In addition, the following conditions must be fulfilled: The signal difference of a particular Probeset must be greater than 40; the change p-value must be below 0.001 for up regulated genes or, alternatively, above 0.999 for down regulated genes.

The comparative analysis of expression data using the Gene Ontology (GO) structured vocabulary was performed with two different programs. For the generation of the GO trees shown in Figure 3 and Figure 5 the lists of regulated genes were imported into the program GoSurfer [20]. In these lists the Affymetrix ProbesetID was used as identifier. For detecting significant categories within the differential expressed genes the gene lists were compared to all Probesets present on the HG_U133A chip. A GO category was considered to be regulated if at least 5 regulated genes were present in the respective category and the p-value was below 0.0001.

For the significance ranking of the GO categories either the up or the down regulated genes were imported into the program EASE [21]. As GeneID the LocusLink numbers were used. In this analysis the following categories were included: GO molecular function, GO biological processes, GO cellular component, KEGG pathway and SwissProt Keyword. A category must contain 4 or more genes with an EASE score of at least 0.001. The EASE score, a derivative of Fisher's Exact probability test was used to determine the significance [21].

For the pathway analysis the program GenMAPP [22] was used. This program allows the visualization of differentially expressed genes on biological pathway maps. The genes that were up regulated in the presence of Fe(II) are represented by shaded boxes, down regulated genes are represented by framed boxes. Clear boxes represent genes that were not significantly affected by Fe(II) addition to the cell culture.

3. Results

To study the effects of processes that could occur at the implant-tissue interface of iron stents we have established a cell culture model. As relevant cells primary vascular smooth muscle cells (SMCs) were isolated from human umbilical cord veins.

3.1. Determination of effective iron concentrations

Microscopic analysis revealed a homogenous spindle shaped morphology expected for SMCs (Fig. 1). First, the concentrations of Fe (II) ions in the culture medium were determined that would affect the phenotype of the cells. To this end, the cells were cultivated in the presence of various concentrations of Fe(II) ions. Depending on the iron concentrations and on the incubation time, the metabolic activity was reduced compared to a culture to which no iron was added (Fig. 2). Concentrations from 0.005 mg/ml to 0.05 mg/ml had similar effects and arrested cell growth. An intermediate concentration of 0.03 mg/ml Fe(II) was used in the subsequent experiments.

3.2. Effect of Fe(II) on DNA synthesis

If the influence of iron ions on the metabolic capacity of cells was due to a decrease in the cell growth rate, then the DNA synthetic rate is expected to be lowered as well. Quantification of the cellular DNA synthesis showed a clear reduction in the presence of Fe(II) which is consistent with a reduced cell proliferation rate (Table 1).

3.3. Gene expression profiling

To investigate the effects of iron in a more global approach, the RNA expression profile was analyzed by gene array hybridization. Random errors were reduced by taking samples at two different time points [23, 24]. Cells were incubated for 12h and for 24h in the presence or absence of Fe(II)(30µg/ml), respectively. Total RNA was extracted, labeled and then hybridized to an oligonucleotide microarray. Signals obtained from individual mRNA species from cells grown in the presence of Fe(II) were compared with control samples and the fold-change calculated. A fold-change of 1.5 was set as a cut-off limit. The analysis was restricted to genes that were consistently regulated in the same direction at both time points (12h vs control and 24h vs control). According to these criteria 557 sequences (Probesets) present on the chip were designated regulated. Increased signals from cells cultivated in the presence of excess Fe(II) were obtained from 251 Probesets, whereas 306 Probesets showed a decreased fluorescence signal intensity.

3.4. Specificity of the cellular response

To determine whether the cellular reaction was specific, the mRNA levels of genes related to iron metabolism were examined. As expected if the cells specifically responded to an excess of iron ions, mRNA levels increased for iron utilizing proteins such as the intracellular iron storage protein ferritin, and the plasma protein ceruloplasmin that oxidizes Fe(II) to Fe (III) (Table 2). The plasma iron storage protein transferrin shows an increase at 12 hours; however, this increase appears only transient since at 24 hours there is a decrease in the transferrin signal intensity. As expected, the transferrin receptor that mediates the cellular iron uptake was down regulated. These results indicate that the cells respond in a specific way to the excess of iron ions.

3.5. Regulation of cellular processes

To identify Fe(II) regulated processes, the gene ontology (GO) was analyzed using gene annotations for processes and molecular functions [25]. Lists of genes that were either up or down regulated were assigned to individual processes. When represented as a hierarchical tree structure, many of the processes that were down regulated in the presence of Fe(II) could be associated with cell proliferation (Fig. 3). The cell cycle and DNA replication were the most significantly down regulated processes (Table 3). These results are in accord with the DNA synthetic rates (Table 1). In agreement with the reduced cell metabolic activity in the presence of Fe(II), some down regulated processes were related to cell proliferation (Table 1 and Fig. 3). To investigate the molecular details a pathway analysis was done [22]. The cell cycle map showed that in the presence of Fe(II) cell cycle genes were down regulated. The cyclin gene (CCN) products form an active complex with the cyclin-dependent kinases (CDKs and CDC2) to promote the entry and progression of the cell cycle. MCM (maintenance of mini-chromosomes) genes are required for DNA replication. p53 is a central inhibitory regulator of the cell cycle. One p53 activity is the transcriptional induction of the gene encoding p21, a key mediator of the p53 cell cycle arrest function. p21 acts by inhibiting the activity of multiple CDKs and shows higher expression in the presence of Fe(II) as expected as a result of increased p53 protein activity (Fig. 4).

In the presence of Fe(II) there were higher levels of mRNA for several genes involved in cell death (Fig. 5). It could therefore not be excluded that the observed decreases in the metabolic activity and in DNA synthesis may have partly been due to cell death. However, the cells had a normal and healthy appearance (Fig. 1). In addition, the cell number based on the metabolic capacity did not decrease even with higher Fe(II) concentrations and during longer incubation periods (Fig. 2). Even though it cannot be excluded that a minor fraction of cells undergo apoptosis in these experiments, these observations argue against programmed cell death of a relevant fraction of the cells. Therefore, the lower metabolic activity and the decrease in DNA synthesis were most likely due to a reduced cell growth rate. Interestingly, Fe(II) caused an increase in the expression of cellular membrane biosynthetic genes (Fig. 5). The affected membrane components included lipids and cholesterol (Table 4). In fact, the overwhelming majority of the cholesterol biosynthetic genes were up regulated, indicating that Fe(II) affects cellular membrane biogenesis (Fig. 6).

4. Discussion

Stent implantation has been demonstrated to result in stimulation of neointima proliferation. This proliferative response is an essential part of the wound-repair process. However, frequently cell proliferation mainly of SMCs continues even after the wound is healed and may eventually lead to a restricted blood flow. To reduce restenosis radioactive stents as well as cytotoxic and anti-inflammatory drug eluting stents have been employed [26].

The data suggests that Fe(II) as a degradation product of iron stents may play a beneficial role by antagonizing SMC proliferation and restenosis. The reduction in cell growth is most likely due to a decreased growth rate rather than being the result of increased cell death as indicated by the normal appearance typical for SMCs and the constant metabolic activity over a prolonged period. An invaluable benefit of using a cell culture model and large DNA arrays is the comparatively unbiased and detailed molecular information of the effects of individual interactions. A large scale expression analysis covering 14,500 genes revealed that a cell cycle master control gene encoding p53 was induced. p53 is a tumor suppressor protein that is induced by various cellular stress conditions, such as ionizing radiation or DNA damage. It acts anti-proliferative and can either arrest the cell cycle to allow DNA repair, or it can induce apoptotic cell death if the damage is so heavy that the repair mechanisms are overwhelmed [27]. Even though p53 can be induced by DNA damage the gene ontology analysis did not show any significant induction of DNA repair processes. p53 activity regulated at the post-transcriptional level by multiple protein-protein interactions, covalent modifications such as phosphorylation, acetylation and sumoylation, as well as sub-cellular localization and eventually proteasome-mediated degradation. In the nucleus p53 acts as a transcriptional activator of various genes involved in diverse functions. p21 is an important anti-proliferative p53 target gene. p21 expression increased in the presence of Fe(II), indicating that p53 activity increases in response to Fe(II). p21 inhibits several protein kinase complexes that are required for cell cycle progression, such as cyclin E-CDK2 or cyclin D-CDK4. The reduced expression of positive regulators of cell proliferation, such as cyclins and CDKs, is in accordance with the cell cycle inhibitory activities of p53 and p21. Therefore, high Fe(II) levels apparently reduce cell growth by affecting cell cycle regulatory gene expression.

Interestingly, Fe(II) induced a vigorous increase in the expression of genes involved in the synthesis of cellular membrane components. Fe(II) could directly act to replace other metal ions or it could affect cellular processes by its reducing power. Alternatively, in the presence of peroxides free or loosely bound Fe(II) can catalyze the formation of radicals such as the hydroxyl radical [28-30]. These effects are not necessarily identical to oxidative stress. Importantly, Fe(II) is a powerful reducing agent and there was no significant increase in gene expression related to oxidative stress. Radicals react vigorously with a wide variety of biomolecules and among these membrane components are of major importance [31-33]. Cholesterol is a determinant of membrane fluidity and plays a role in the function of membrane proteins. It is reasonable and consistent with published data to hypothesize that ferrous ion induced radical generation leads to membrane disturbances and in a compensatory response the cells may attempt to increase the cholesterol synthetic capacity [34-36]. In reports published so far the Fe(II)-dependent effects on cholesterol metabolism vary depending on the experimental system used. However, in support of the above hypothesis antioxidants suppressed the Fe(II) ion induced cholesterol synthesis in a cell culture model [37].

5. Conclusion

A cell culture model has been established to investigate molecular details of a specific interaction between vascular cells and an iron degradation product. The down regulation of cell proliferation presence of excess Fe(II) suggest that specific iron stent degradation products could have a beneficial effect in the control of neointima proliferation. Gene expression profiling has yielded detailed insights in the mechanisms of Fe(II) mediated cellular responses. Most importantly, in the presence of Fe(II) the expression level was higher for the key cell cycle regulator p53 as well as for cholesterol synthetic enzymes. This information could be used to identify strategies to optimize the performance of iron stents, such as the application of iron chelators or inhibitors of cholesterol synthesis.

Acknowledgements

We gratefully acknowledge the highly skilled technical help of Franziska Dimpfel and Tanja Töpfer, as well as the generous support of Robert Geffers with the microarray analysis. This work was supported by the DFG (SFB 599) and by the European Community (QLG2-CT-2000-00345 and QLG2-CT-2000-00930).

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