High resolution imaging of collagen organisation and synthesis using a versatile collagen specific probe

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Abstract

Collagen is the protein primarily responsible for the load-bearing properties of tissues and collagen architecture is one of the main determinants of the mechanical properties of tissues. Visualisation of changes in collagen three-dimensional structure is essential in order to improve our understanding of collagen fibril formation and remodelling, e.g. in tissue engineering experiments. A recently developed collagen probe, based on a natural collagen binding protein (CNA35) conjugated to a fluorescent dye, showed to be much more specific to collagen than existing fluorescent techniques currently used for collagen visualisation in live tissues. In this paper, imaging with this fluorescent CNA35 probe was compared to imaging with second harmonic generation (SHG) and the imaging of two- and three-dimensional collagen organisation was further developed. A range of samples (cell culture, blood vessels and engineered tissues) was imaged to illustrate the potential of this collagen probe. These images of collagen organisation showed improved detail compared to images generated with SHG, which is currently the most effective method for viewing three-dimensional collagen organisation in tissues. In conclusion, the fluorescent CNA35 probe allows easy access to high resolution imaging of collagen, ranging from very young fibrils to more mature collagen fibres. Furthermore, this probe enabled real-time visualisation of collagen synthesis in cell culture, which provides new opportunities to study collagen synthesis and remodelling.

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1. Introduction

Living tissues are composed of cells embedded in extracellular matrix (ECM), the latter mainly consisting of proteoglycans, collagen and elastin. Collagen is the main load-bearing component within the tissue, while the elastin provides elasticity to the tissue and the proteoglycans give the tissue its swelling capacity. In load-bearing tissues collagen is abundantly present and the mechanical properties depend on the collagen fibre architecture, e.g. collagen fibre orientation, collagen fibre content and collagen fibre cross-linking (Billiar and Sacks, 2000a,b; Dahl et al., 2005). Studying the active change in collagen architecture is the focus of diverse fields of research, including developmental biology, biomechanics and tissue-engineering. Progress in these fields requires further elucidation of collagen fibril formation and remodelling processes by imaging the local three-dimensional (3D) collagen organisation.

Collagen fibres and bundles can be visualised in living tissues without the use of specific probes, through techniques such as polarised light, phase contrast microscopy (de Campos Vidal, 2003) and differential interference contrast microscopy (Petrill et al., 2004). Techniques for 3D...
visualisation of collagen without the use of specific probes include autofluorescence (Voytik-Harbin et al., 2001; Zipfel et al., 2003), confocal reflection microscopy (Hartmann et al., 2006; Voytik-Harbin et al., 2001; Brightman et al., 2000; Wolf and Friedl, 2005) and second harmonic generation (SHG) using femtosecond pulsed infrared excitation (Cox et al., 2003; Campagnola et al., 2002; Zoumi et al., 2002; Konig and Riemann, 2003). Several tissue constituents possess intrinsic autofluorescence properties, enabling them to be visualised using confocal or multi-photon microscopy without the use of added probes. However, autofluorescence is not specific enough and autofluorescence intensity is relatively low, especially when it is used in combination with fluorescent probes (Zipfel et al., 2003; Richards-Kortum and Sevick-Muraca, 1996). Similarly, with confocal reflection microscopy it is difficult to discriminate between the constituents in cultured tissue constructs due to the absence of spectral information. SHG is only expressed by ordered non-centrosymmetric materials (e.g. collagen) and is used to detect the orientation and distribution of mature and immature collagen fibril segments (Williams et al., 2005; Konig et al., 2005). The strong forward scattered SHG (Williams et al., 2005; Cox et al., 2003; Campagnola et al., 2002) enables detailed visualisation of collagen organisation within various tissues. However, depending on the tissue properties forward scattered SHG is not always feasible. Furthermore, SHG requires high laser power, which increases the risk of collateral damage to cells and tissue.

In view of the shortcomings of these existing techniques, we recently developed a collagen specific fluorescent probe (Krahn et al., 2006). This fluorescent CNA35 probe consists of a part of a bacterial collagen binding protein domain (CNA35), which is covalently bound to a commercially available fluorescent dye. Using solid phase binding assays and immunohistochemical staining we previously showed that this probe was more specific for collagen than dichlorotriazinyl aminofluorescein (DTAF) (Krahn et al., 2006). The fluorescent CNA35 probe showed affinity for both fibrillar and non-fibrillar collagen. In this study we continue the development of this fluorescent CNA35 probe. By comparing it directly to SHG and applying it to a wide variety of samples the use of this fluorescent probe at different structural levels is demonstrated. We show that this probe reveals more detail in the collagen organisation compared to SHG, including visualisation of very small collagen fibrils. Furthermore, the probe allows for real-time monitoring of collagen synthesis, thus enabling us to study the active change in collagen organisation.

2. Methods

2.1. Fluorescent CNA35 collagen probe

A vector coding for the collagen binding domain A of Staphylococcus aureus was transformed into Escherichia coli and expression of this collagen binding domain was induced, as described in Krahn et al. (2006). This domain (CNA35) was purified and subsequently labelled with either Oregon green 488 (CNA35-OG488; Invitrogen, The Netherlands) or Alexa Fluor 488 (CNA35-AF488; Invitrogen, The Netherlands). The fluorescent CNA35 probe was applied to four different sample types.

2.2. Imaging a mouse carotid artery

A Swiss mouse was euthanised by a mixture of O2/CO2 gas. Carotid arteries were isolated and mounted on two glass micropipettes in a perfusion chamber (Hilgers et al., 2003; Megens et al., 2007b) filled with 10 ml phosphate buffered saline (PBS; Sigma, USA) containing the fluorescent probe(s). A transmural pressure of 80 mm Hg was applied in order to mimic physiological pressure. Experiments were approved by the local ethics committee on the use of laboratory animals. Procedures followed were in accordance with the institutional guidelines.

SYTO44 (Invitrogen, the Netherlands), eosin (Invitrogen, the Netherlands), and CNA35-OG488 were used as specific fluorescent markers for DNA/RNA, elastin, and collagen, respectively (van Zandvoort et al., 2004). All probes were applied extraluminally, were excitable with two-photon microscopy, and exhibited emission spectra with maxima at 480, 560, and 520 nm, respectively. For comparison with SHG, the labelling solution contained CNA35-OG488 [1.0 μM] in PBS. For imaging of the carotid artery, a mixture of SYTO44 [1.5 μM], CNA35-OG488 [1.0 μM] and eosin [0.25 μM] in PBS was used.

For imaging a Nikon E600FN upright microscope (Nikon Corporation, Japan), coupled to a standard Biorad 2100 MP multiphoton system (Biorad, Great-Britain) was used (van Zandvoort et al., 2004). A 140-fs pulsed Ti: sapphire laser (Spectra Physics Tsunami, USA) was tuned and mode-locked at either 800 nm (fluorescence) or 840 nm for SHG. A 60× water dipping objective with a 2.0 mm working distance was used for imaging in upright geometry (numerical aperture (NA) 1.0, Nikon). For the SHG experiment, two photomultiplier tubes (PMT) were used to detect the emitted (fluorescent and SHG) signals. The channel of PMT1 was tuned at 400–500 nm in order to detect SHG and the channel for PMT2 was tuned at 500–560 nm for detecting the CNA35-OG488. For imaging of the carotid artery using the combination of fluorescent markers, three PMTs were used. The channels of the three PMTs were tuned as follows: 470–480 nm, SYTO44 (PMT1); 500–520 nm, CNA35-OG488 (PMT2); 590–610 nm, eosin (PMT3). Separate images were obtained from each PMT (coded blue, green, and red, respectively) and combined into a single image.

2.3. Imaging an engineered cardiovascular construct

Human vena saphena (HVS) myofibroblasts were obtained from patients and expanded using regular cell
Cell culture methods (Schnell et al., 2001). Cell culture medium consisted of advanced Dulbecco’s Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Biochrom, Germany), 1% L-glutamax (Gibco, USA) and 0.1% gentamycin (Biochrom, Germany). A rectangular shaped (0.5 × 2.5 × 1.0 mm³) non-woven polyglycolic acid (PGA) scaffold (density 72.76 mg/cm²; Cellon, Luxemburg) was coated with 1% (w/v) poly-4-hydroxybutyrate (P4HB; Symetis Inc., Switzerland) in tetrahydrofuran (THF; Merck, Germany) and 0.1% gentamycin (Biochrom, Germany) were used as specific fluorescent markers for cell cytoplasm and collagen, respectively. AF488 was used because of its superior photostability compared to OG488. The emission peaks of the CTO and CNA35-OG488 are situated at 520 nm and above 560 nm. Labelling of the tissue was performed with tissue culture medium, which was supplemented with CTB [0.475 μM] and CNA35-AF488 [3.0 μM]. The CTB solution was applied for 5 h, followed by CNA35-OG488 [3.0 μM] for 16 h. The samples were then placed in tissue culture medium.

An inverted Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) was used to image the tissue engineered construct. A chameleon ultra 140-fs pulsed Ti:Sapphire laser (Coherent, USA), was tuned to 760 nm to excite using both SHG (A) and the collagen probe (B). The image obtained using both SHG (A) and the collagen probe (B) of the probe for the collagen fibres. The image obtained using both SHG (A) and the collagen probe (B). The image obtained using both SHG (A) and the collagen probe (B). The image obtained using both SHG (A) and the collagen probe (B). The image obtained using both SHG (A) and the collagen probe (B). The image obtained using both SHG (A) and the collagen probe (B).

Cell Tracker Orange CMRA (CTO; Invitrogen, the Netherlands) was used as a specific fluorescent marker for cell cytoplasm. The CTO and CNA35-OG488 were excited using a Helium–Neon laser (488 nm) and an Argon Laser (543 nm), respectively. Two PMTs were used and the channels were tuned as follows: 505–530 nm, CNA35-OG488 (PMT1); 585 nm and longer, CTO (PMT2). After excitation, the cells were rested for 24 h. After these 24 h the incubator setup was placed on the microscope and the cell culture was subsequently studied for 52 h with the labelling solution present. The microscope settings (gain, laserpower) were based on a 48 h old culture, corresponding to 24 h after the start of imaging. This was done to prevent saturation of the signal with increase in collagen content while simultaneously allowing the detection of sufficient detail early on in the culture. Images were recorded every 60 min. Furthermore, using an identical experimental setup another 24 h old cell culture was used to image a single cell and its surrounding collagen.

3. Results

The fluorescent CNA35 probe was applied to several types of cultures in order to evaluate its imaging properties for collagen.

Fig. 1 shows an image of the collagen organisation in the tunica adventitia (outer layer of the artery) of a structurally intact (non-viable) mouse carotid artery, obtained using both SHG (A) and the collagen probe (B). The signal originating from the CNA35-OG488 bound to the collagen fibres in the mouse carotid artery aligns perfectly with the SHG signal, which originates from the collagen fibres in the same focal plane and confirms the specificity of the probe for the collagen fibres. The image obtained using the CNA35-OG488 shows much more fine structures compared to the image obtained with SHG. At greater depth, the intensity of the emitted CNA35 signal is higher, as is apparent from the fact that a much lower laser power (5% versus 40%) is needed to generate signals of similar strength using two-photon LSM and SHG, respectively.
In Fig. 2A a schematic overview of a structurally intact carotid artery is shown, which also illustrates the origin of the consecutive two-photon images (x/y-scans, B–E). In these images collagen fibrils (yellow), cell nuclei (blue) and elastin (red) are shown. (B) and (C) show the tunica adventitia, which consists primarily of collagen fibrils, fibroblast-like cell nuclei and a few elastin fibres. The tunica media (D–E) consists of collagen, elastin and smooth muscle cell (SMC) nuclei. SMCs are primarily oriented perpendicular to the longitudinal axis of the artery. Small collagen fibrils are clearly visible in between the smooth muscle cell nuclei. Note that collagen and elastin are distinguishable.

In order to show that the probe enables visualisation of very small and young collagen fibrils, we imaged an engineered cardiovascular construct of approximately 1 mm thickness after three weeks of culture. Fig. 3 and its Supplementary movie show detailed two-photon images of the constituents of the engineered tissue constructs: HVS cells (blue), collagen fibrils (green) and PGA/P4HB scaffold (purple). The collagen fibrils are in an early stage of development and are still very thin compared to the collagen fibrils in the tunica adventitia (Fig. 1). Collagen fibrils typically have a diameter in between 10–500 nm (Fratzl, 2003; Ushiki, 2002). Notably, the collagen fibrils show a wavy pattern, as is observed in native collagenous tissues, and the fibrils are closely associated with the surface of the cells (indicated with arrows in Fig. 3). This close association to the cell surface is most clearly seen in the Supplemented movie.

In order to demonstrate the opportunities of this fluorescent CNA35 probe for high resolution imaging, the probe was applied to a cell culture. Zooming in to a single cell (Fig. 4), we can see very detailed collagenous microstructures (green) originating from the cell (red). In red the cell cytosol is visible, which clearly outlines the darker cell nucleus and the cell organelles. Very small collagen fibres and fibre networks are distributed over the entire imaging plane surrounding the cell. The collagen probe is also attached to very small and round structures within the cell cytosol, which are not observed in the cell nucleus. These structures are most likely the result of pinocytosis, in which endocytic vesicles non-specifically take up small droplets of extracellular fluid and any material dissolved in it (Lodish et al., 1995). Another possibility could be the existence of fibropositors of collagen, as was suggested by Canty and Kadler (2005).

To study its applicability in real-time imaging of collagen fibril formation, CNA35-AF488 was used in a cell culture experiment. Fig. 5A–F show six confocal images from the same spatial and focal position obtained over a period of 52 h. In the images and Supplementary movie interesting areas are indicated with white circles. These images and especially the Supplemented movie show that the cells (red) move around and actively secrete collagen (green) into their surroundings. The first image shows a very limited amount of collagen, which could be either newly formed or still attached to the cell and left over from the previous culture. Over time the collagen content increases and fibre-like structures start to appear, which is very clearly shown within the white circles. Some cells move around with the collagen fibres closely attached to their surface and form larger collagen aggregates, other cells release the collagen into the surroundings. Most importantly the images show the ability to monitor changes in collagen organisation over time. However, several newly synthesised collagen fibres remain detached from the cell surface and float in the culture surroundings. In order to demonstrate if CNA35 affected cell-collagen interaction, collagen type I coated culture plates were seeded with myo-
In a long-term experiment no effect on cell attachment was observed and in a short-term experiment an increase in attachment was observed. In addition, in the presence of high concentrations of CNA35-OG488 (3.0 μM), tissue engineered cardiovascular constructs showed similar compaction to unstained tissue engineered constructs. These data suggest that there is no negative effect of CNA35 on cell-collagen interaction.

4. Discussion

In this paper we further explored the performance of a recently developed collagen specific probe (Krahn et al.,...
The fluorescent CNA35 probe was applied to samples with different levels of maturity and demonstrated improved properties with regard to visualisation of different structural levels of collagen organisation. The probe showed an improvement in detail and enabled real-time monitoring of collagen synthesis.

A mouse carotid artery was imaged using both SHG and fluorescently labelled CNA35. Fig. 1A and B show that the signal from the probe colocalises with the SHG signal, which confirms the collagen specificity of the probe (Krahn et al., 2006). Furthermore, it shows that the CNA35 probe reveals much more detail (fine structures) than the backscattered SHG signal. This can possibly be explained by difference in signal strength obtain with SHG and imaging with fluorescent CNA35. The small fibrils probably do not generate enough SHG to be visualised, whereas with fluorescent enhancement this is possible. Image formation with SHG and fluorescent probes using two-photon laser scanning microscopy is different (Gauderon et al., 2001; Moreaux et al., 2001), e.g. resulting in differences in polarisation information. Furthermore, SHG can only be generated in non-centrosymmetric assemblies of chiral molecules. SHG is strongest in forward scatter geometry (Williams et al., 2005; Cox et al., 2003; Konig et al., 2005), however, in certain dense and thick tissues (e.g. large arteries) it is difficult to obtain images with the forward scattered SHG of collagen fibres (Boulesteix et al., 2006; Megens et al., 2007b). This limits the use of forward scattered SHG and requires additional techniques to image collagen in backward geometry, which is the geometry used in this study. Apparently, the SHG in backward geometry is much weaker than the fluorescence signal of the collagen probe. The stronger signal of the collagen probe enables us to use lower laser power for imaging, which reduces the risk of tissue destruction and allows the use of a wide range of laser powers to image deeper into the tissue. In tissue engineered constructs, images have been recorded 110 μm deep and imaging was not limited by the diffusion of the probe. In addition, Megens et al. (2007a) applied the CNA35-OG488 probe to muscular and elastic arteries and showed limited labelling in viable elastic arteries by the presence of intact endothelium and elastic laminae. This demonstrated that the availability of the probe in specific tissue regions depends on the tissue composition, the concentration of the applied probe, the amount of time the probe is applied to the tissue and the way the probe is applied to the tissue (e.g. intra- and extraluminally). Microtubules and skeletal muscle exhibit SHG as well, albeit at a much lower signal compared to collagen (Zipfel et al., 2003). The probe has been tested for specificity by using solid phase binding assays (Krahn et al., 2006) and immunohistological staining, which suggested that the probe binds specifically to collagen with a different affinity for the different collagen types (I–VI). A possible drawback to the use of fluorophores is the risk of photo-bleaching, whereas intrinsic tissue properties like SHG do not exhibit photo-bleaching.

In addition, an artery was stained to visualise the cells, the elastin network and the collagen network. A detailed image of the collagen organisation within the tissue is obtained, showing images of collagen fibres ranging from a few microns thick (~4.0 μm) to sub-micron resolution.
The collagen probe revealed small collagen fibres in between the smooth muscle cells in the tunica media of the carotid artery, which are much more difficult to observe with the use of SHG (Zoumi et al., 2004; Boulesteix et al., 2006; Schenke-Layland et al., 2005, 2006).

The probe was applied on three weeks old tissue engineered cardiovascular constructs in order to show its usefulness in visualizing the developing collagen fibrils within live young tissues. After three weeks of culture the construct predominantly consists of cells, glycosaminoglycans, collagen and PGA scaffold (Mol et al., 2005). The cells, the collagen and the PGA scaffold were imaged. Fig. 3 shows that the collagen fibrils of the tissue engineered construct are relatively thin compared to fibrils in mature native artery (Figs. 1 and 2) and that these fibrils exhibit a very diffuse organisation compared to native fibrils. Mature native fibrils are bundled in fibres rather than in individual fibrils. The benefit of the fluorescent probe used here is that it binds to individual collagen fibrils (Zong et al., 2005) and allows collagen fibrils with sub-resolution diameter to be imaged. Collagen fibres typically have a diameter in between 10–500 nm (Ushiki, 2002). Several successful attempts have been performed to study ECM remodelling in time by using confocal reflection microscopy in 3D fibrin lattices and fibroblast populated collagen lattices (Hartmann et al., 2006; Brightman et al., 2000; Voytik-Harbin et al., 2001; Wolf and Friedl, 2005). However, with confocal reflection microscopy alone it is not possible to distinguish between different constituents, whereas with multiple fluorescent labels in combination with laser scanning microscopy this is possible. Collagen assembly by cells has previously been quantified using FITC labelled rat-tail collagen (Johnson and Galis, 2003) to monitor the remodelling and structure of exogenous collagen. The study presented here is the first to look at fluorescently enhanced endogenous collagen.

Fig. 4 show that the probe enables detailed visualisation of collagen structures at high magnification and provides new opportunities to visualise collagen at the cellular level. The fluorescent collagen probe also allows real-time visualisation of collagen synthesis. This was demonstrated by seeding myofibroblast cells on top of a glass coverslip and studying these cells over time in the presence of the probe. The probe was coupled to a more stable fluorescent dye to prevent possible bleaching of the probe. Myofibroblasts are involved in wound healing and pathological processes and predominantly synthesise type I and type III collagen (Hinz et al., 2001). The real-time collagen synthesis shows similar features to real-time elastin fibrillogenesis, which has been studied with confocal laser scanning microscopy (Kozel et al., 2006; Czirok et al., 2006). One can see collagen fibrils that are closely associated to the cell surface and that are subsequently transferred to other extracellular collagen fibres. However, more detailed studies have to be performed to elucidate the exact process of collagen fibre formation. Potential drawbacks to the collagen probe are its effects on cell-collagen interaction and fibril formation. However, the collagen probe did not show any negative effect on cell-collagen interaction. A theoretical drawback of this probe is that it binds directly to the collagen. By binding to the collagen the probe potentially prevents proper fibril formation. Krahni et al. (2006) show that the dissociation constant ($K_d$) of CNA35 is approximately $10^{-7}$–$10^{-6}$, which ensures that the binding is sufficiently strong but not irreversible. When medium containing CNA35-OG488 is removed from the cell culture and replaced with plain culture medium, a new equilibrium will form between the probe bound to the collagen and the plain culture medium. Due to the absence of probe in the culture medium this equilibrium will shift towards the culture medium, which effectively decreases the concentration of probe bound to collagen.

In summary the present study has shown that the CNA35-based collagen probe enables high resolution imaging of collagen organisation and synthesis in great detail. The probe can be used to study very young and thin collagen fibrils in cell culture and more mature collagen in native tissue. Furthermore, this study is the first to report on real-time monitoring of collagen synthesis. The use of fluorescent CNA35 for imaging of collagen results in more detail compared to SHG while using lower laser powers. The probe is very flexible in its design. Different probes (e.g. fluorescent, MRI) can be coupled to the collagen binding domain, which provides new possibilities to study collagen organisation and remodelling with high resolution.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2007.04.008.

References


