AOP 173 on Substance interaction with the lung resident cell membrane components leading to lung fibrosis

Verification of AOP readiness for WNT and WPHA submission

The EAGMST Subgroup on AOP external review was consulted to conduct the verification of AOP 173, which had been revised following the scientific review conducted in 2020. The task was accepted by Rex FitzGerald.

The external review subgroup and any interested EAGMST members were invited to join a TC organised on 25 March 2022 to discuss the outcome of the verification.

Participant in the TC:

External review subgroup (including ad hoc participants)	**AOP 173 authors**
Rex FitzGerald Shihori Tanabe Yhun Yhong Sheen Carole Yauk Nathalie Delrue	Sabina Halappanavar

Outcome of the TC:

The authors of AOP 173 provided, ahead of the verification, a document where the comments from the review panel are addressed extensively, and corresponding revisions brought to the AOP are also shown. Based on the review of these responses, Rex was confident that the AOP has been adequately revised. This was supported by the other participants in the TC. The group commended the authors for a very thorough revision. It was acknowledged that all comments have not led to revisions of the AOP but the group supported the AOP authors in their rationales, in particular regarding their choice of the MIE and the role of ROS. Considering that AOPs are linear descriptions of biological events, it was agreed that ROS could be included as additional KE(s) but not in this AOP. During the TC, it was also agreed that Sabina Halappanavar would work with Brigitte Landesmann to merge KE 1501 (of AOP 173) *Increased, extracellular matrix deposition* with a KE already in the Wiki describing the same process: KE 68 *Accumulation, Collagen*. This has now been done and AOP 173 includes KE 68. The gardeners have been informed that KE 1501 can be deleted.

TC follow up actions:

The follow up actions identified at the TC are listed below:

- Update the AOP title to make it consistent with the MIE title
- Merge the KE as suggested by Brigitte Landesmann – i.e. (i) work on the KE with Brigitte ensuring adequate merging (see annex), (ii) inform co-authors that the current KE will not be used anymore, (iii) make all necessary adaptations for KE 68 to be used in the AOP, (iv) inform the gardeners that the other KE can be deleted, if cannot be done by author.

As of 18 July 2022, the auhtors have completed the follow-up actions described above, except (ii) and (iv). For these two, they will wait until endorsement of the AOP.

Annex: KE 68: Accumulation, Collagen

Collagen is mostly found in fibrous tissues such as tendons, ligaments and skin. It is also abundant in corneas, cartilage, bones, blood vessels, the gut, intervertebral discs, and the dentin in teeth. In muscle tissue, it serves as a major component of the endomysium. Collagen is the main structural protein in the extracellular space in the various connective tissues, making up from 25% to 35% of the whole-body protein content. In normal tissues, collagen provides strength, integrity, and structure. When tissues are disrupted following injury, collagen is needed to repair the defect. If too much collagen is deposited, normal anatomical structure is lost, function is compromised, and fibrosis results.

The fibroblast is the most common collagen producing cell.Collagen-producing cells may also arise from the process of transition of differentiated epithelial cells into mesenchymal cells (EMT). This has been observed e.g. during renal fibrosis (transformation of tubular epithelial cells into fibroblasts) and in liver injury (transdifferentiation of hepatocytes and cholangiocytes into fibroblasts) (Henderson and Iredale, 2007).

There are close to 20 different types of collagen found with the predominant form being type I collagen. This fibrillar form of collagen represents over 90 percent of our total collagen and is composed of three very long protein chains which are wrapped around each other to form a triple helical structure called a collagen monomer. Collagen is produced initially as a larger precursor molecule called procollagen. As the procollagen is secreted from the cell, procollagen proteinases remove the extension peptides from the ends of the molecule. The processed molecule is referred to as collagen and is involved in fiber formation. In the extracellular spaces the triple helical collagen molecules line up and begin to form fibrils and then fibers. Formation of stable crosslinks within and between the molecules is promoted by the enzyme lysyl oxidase and gives the collagen fibers tremendous strength (Diegelmann,2001). The overall amount of collagen deposited by fibroblasts is a regulated balance between collagen synthesis and collagen catabolism. Disturbance of this balance leads to changes in the amount and composition of collagen. Changes in the composition of the extracellular matrix initiate positive feedback pathways that increase collagen production.

Normally, collagen in connective tissues has a slow turn over; degradating enzymes are collagenases, belonging to the family of matrix metalloproteinases (MMPs).Other cells that can synthesize and release collagenase are macrophages, neutrophils, osteoclasts, and tumor cells (Di Lullo et al., 2001; Prockop and Kivirikko, 1995; Miller and Gay, 1987;Kivirikko and Risteli, 1976).

How It Is Measured or Detected

Determination of the amount of collagen produced in vitro can be done in a variety of ways ranging from simple colorimetric assays to elaborate chromatographic procedures using radioactive and non-radioactive material. What most of these procedures have in common is the need to destroy the cell layer to obtain solubilized collagen from the pericellular matrix. Rishikof et al describe several methods to assess the in vitro production of type I collagen: Western immunoblotting of intact alpha1(I) collagen using antibodies directed to alpha1(I) collagen amino and carboxyl propeptides, the measurement of alpha1(I) collagen mRNA levels using real-time polymerase chain reaction, and methods to determine the transcriptional regulation of alpha1(I) collagen using a nuclear run-on assay (Rishikof et al., (2005) .

Histological staining with stains such as Masson Trichrome, Picro-sirius red are used to identify the tissue/cellular distribution of collagen, which can be quantified using morphometric analysis both in vivo and in vitro. The assays are routinely used and are quantitative.

Sircol Collagen Assay for collagen quantification:

The Serius dye has been used for many decades to detect collagen in histology samples. The Serius Red F3BA selectively binds to collagen and the signal can be read at 540 nm (Chen & Raghunath, 2009; Nikota et al., 2017).

Hydroxyproline assay:

Hydroxyproline is a non-proteinogenic amino acid formed by the prolyl-4-hydroxylase. Hydroxyproline is only found in collagen and thus, it serves as a direct measure of the amount of collagen present in cells or tissues. Colorimetric methods are readily available and have been extensively used to quantify collagen using this assay (Chen & Raghunath, 2009; Nikota et al., 2017).

Ex vivo precision cut tissue slices

Precision cut tissue slices mimic the whole organ response and allow histological assessment, an endpoint of interest in regulatory decision making. While this technique uses animals, the number of animals required to conduct a dose-response study can be reduced to $1/4^{th}$ of what will be used in whole animal exposure studies (Rahman et al., 2020).

References

Chen, C. and Raghunath, M. (2009). Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis _ state of the art. Fibrogenesis & Tissue Repair, 2(1).

Nikota, J., Banville, A., Goodwin, L., Wu, D., Williams, A., Yauk, C., Wallin, H., Vogel, U. and Halappanavar, S. (2017). Stat-6 signaling pathway and not Interleukin-1 mediates multi-walled carbon nanotube-induced lung fibrosis in mice: insights from an adverse outcome pathway framework. Particle and Fibre Toxicology, 14(1).

Rahman, L., Williams, A., Gelda, K., Nikota, J., Wu, D., Vogel, U., & Halappanavar, S. (2020). 21st Century Tools for Nanotoxicology: Transcriptomic Biomarker Panel and Precision-Cut Lung Slice Organ Mimic System for the Assessment of Nanomaterial-Induced Lung Fibrosis. Small, 16(36), e2000272