Collagen quantification in cell cultures and tissues

Reinout Stoop[1]
Jessica Snabel[1]
Karin Toet[1]
Marion König[1]
Natascha van Lent[2]
Jan Verheijen[2]
Roeland Hanemaaijer[1,2]

[1]TNO Metabolic Health Research
Leiden, The Netherlands
[2]QuickZyme Biosciences B.V.
Leiden, The Netherlands

Roeland.Hanemaaijer@TNO.nl

TNO innovation for life

Introduction

Reliable quantification of extracellular matrix production is of key importance in fibrosis research, with collagen being one of the major matrix components. The Sirius Red based collagen assay is a widely used assay for collagen quantification and based on precipitation of soluble or solubilized collagen. Sirius Red is known to bind fibrillar collagens. Another commonly used assay is the hydroxyproline based collagen assay. Hydroxyproline based collagen assays measure hydroxyproline in hydrolyzed samples. We studied the applicability of these assays for collagen quantification in both cell culture and tissues.

Methods

For in vitro experiments human primary stellate cells were grown for 4 days in the presence of 20 ng/ml TGFß. Conditioned culture media were collected and collagen was determined using two Sirius Red-based assays (Biocolor Sircol assay (BC) and QuickZyme Soluble Collagen assay (QZ)) and a hydroxyproline-based assay (QuickZyme Total Collagen assay). BC recognizes both fibrillar and unfolded collagen, whereas QZ only recognizes fibrillar collagen. In addition, in pellets from the Sirius Red based assays also hydroxyproline was analyzed using the Total Collagen assay.
Mouse tissues were taken from C57Bl/6 mice. Upon sacrifice tissues were ground in liquid nitrogen, and the powdered tissue used in extraction experiments. Extraction was performed using either 0.5M HAc, 0.5M HAc+pepsin, 1% SDS or 0.5M lactic acid (o/n, 4°C). Collagen was quantified both in the total tissue and the solubilized fraction using hydroxyproline analysis. Liver fibrosis was induced by intraperitoneal injection of CCl_4, which was given 3 times a week. Mice were sacrificed at day 28 or day 42.

Results cell cultures

In vitro fibrosis experiments were performed by TGFß-stimulation of stellate cells. It was observed that collagen production was induced upon TGFß-stimulation (Fig. 1). Sensitivity could be increased by hydroxyproline analysis of the Sirius Red pellet (direct hydrolysis data from control cells were below assay sensitivity).

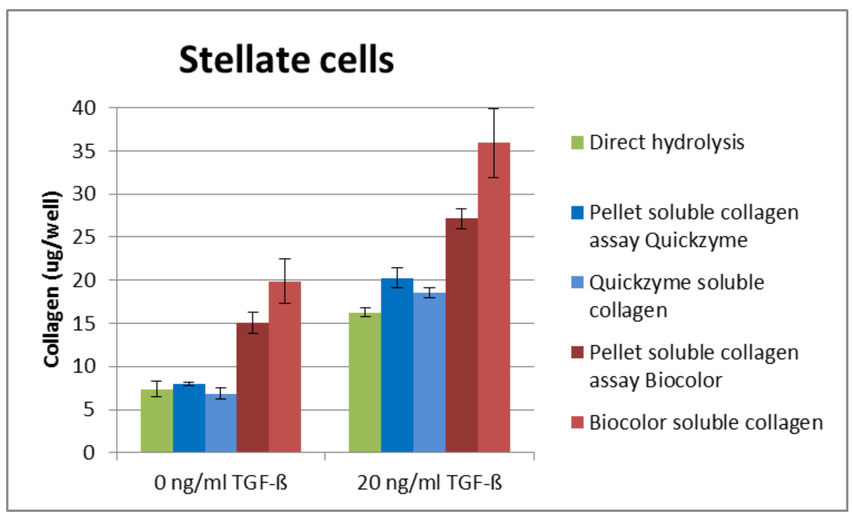

Figure 1: *Collagen analysis in conditioned culture medium of TGFß- stimulated human stellate cells using Sirius Red based collagen assay (QZ or BC) and colorimetric hydroxyproline analysis (upon hydrolysis of medium). In addition hydroxyproline analysis was performed on the Sirius Red pellets of the QZ and BC assay.*

QuickZyme Biosciences

TNO innovation for life

Results tissues

Extraction from powdered tissue using HAc showed that only a minor part of the collagen was solubilized (Table 1). Also other ways of solubilization showed poor efficiency, both in healthy tissue and fibrotic tissue (Fig. 2)

	total collagen (µg per mg wet tissue)	solubilized collagen (µg per mg wet tissue)	% solubilized
lung	11.7	0.3	2
kidney	6.7	0.6	9
heart	3.9	1.1	27
skin	133.3	3.6	3
bovine tendon	400.5	1.1	0

Table 1: *Extraction efficiency of collagen from various tissues.*
Collagen (hydroxyproline) was analyzed in both tissues and acetic acid/pepsin solubilized (o/n 4°C) fractions.

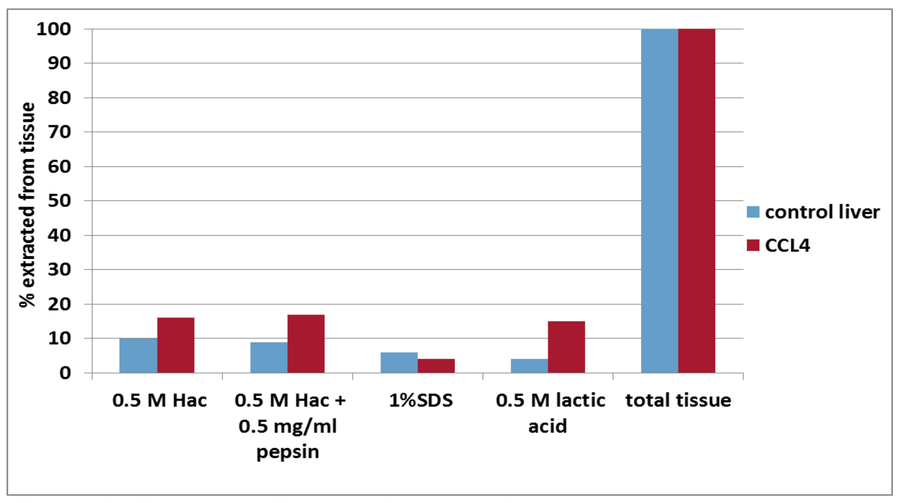

Figure 2: *Extraction efficiency of collagen from liver tissue using various extraction solutions*
Collagen (hydroxyproline) was analyzed in both tissues and the solubilized fractions.
100% control liver: 1.4 µg collagen/mg wet tissue; fibrotic liver: 3.4 µg collagen/mg wet tissue

If extraction was performed at 37°C collagen could be extracted using HAc. However, the collagen was not fibrillar anymore (probably unfolded) and was detectable only using hydroxyproline based collagen assay (Fig. 3).

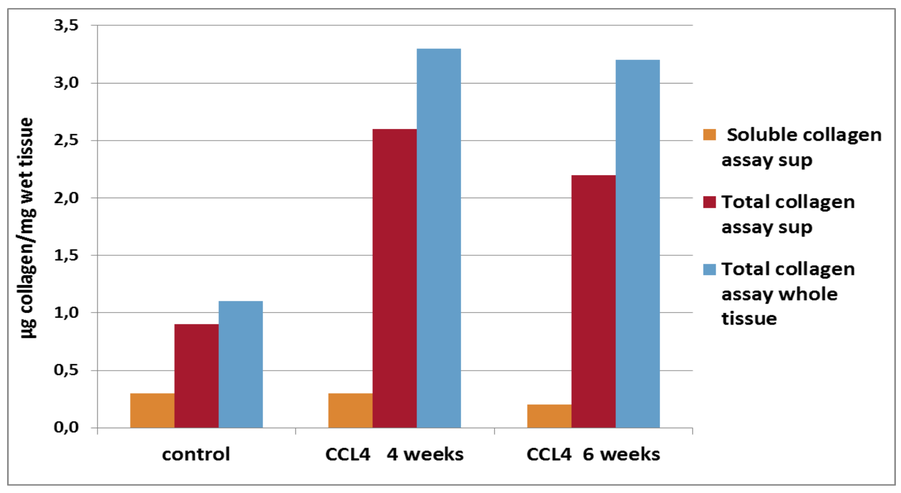

Figure 3: *Extraction efficiency of collagen from liver tissue at 37°C*
Collagen was analyzed in both tissue (hydroxyproline) and in HAc solubilized fractions (Sirius Red (QZ) and hydroxyproline based assay)

Conclusions

- **Extraction efficiency of collagen from tissues is poor (5-30%) -> Collagen quantification in tissues is best done by tissue hydrolysis followed by hydroxyproline analysis.**
- **Extraction efficiency is increased at 37°C, but the extracted collagen is no longer in fibrillar form, and only detectable using hydroxyproline-based assays**
- **TGFß-stimulated human primary stellate produce collagen. Two commercial Sirius Red based assays give different results.**