LC-MS/MS ANALYSIS OF THE EXTRACELLULAR MATRIX OF CULTURED FIBROBLASTS

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INTRODUCTION

Hair follicles undergo growth cycles. Dermal papilla is a compartment localized at the basis of each follicle, and is specifically able to induce the growth of a hair shaft. It is mainly composed of a network of proteins, the extracellular matrix (ECM), in which specialized fibroblasts are scattered. The characterization of the protein composition of the ECM of dermal papilla is likely to contribute to a better understanding of the specific activity of that structure.

We have developed the coupling between liquid chromatography and tandem mass spectrometry (LC-MS/MS) for characterizing the proteins of the ECM synthesized by human fibroblasts. First, the proteins of ECM prepared from cultured dermal fibroblasts were separated by SDS-PAGE and digested by trypsin, to be analysed by LC-MS/MS. Starting from only 8 µg protein, a few tens of proteins could be identified. Besides a certain number of proteins known to be constitutive of ECM, our analyses led to the identification of: (i) membrane-bound proteins; (ii) soluble proteins possibly present within the ECM, to exert different kinds of activities; (iii) proteins constitutive of the cytoskeleton; (iv) proteins of unknown function. Three cultures of fibroblasts were analysed to evaluate the reproducibility of protein identifications. We assessed the contribution of two parameters to the variability of the identifications: the preparation of the protein sample on the one hand (matrix extraction, SDS-PAGE and enzymatic digestion); the LC-MS/MS analysis on the other hand. We are currently developing a multidimensional liquid chromatography approach (2DLC-MS/MS). It consists of analysing the protein sample digest on two chromatographic columns in series. This strategy is likely to provide complementary protein identifications, with an overall gain in analysis time.

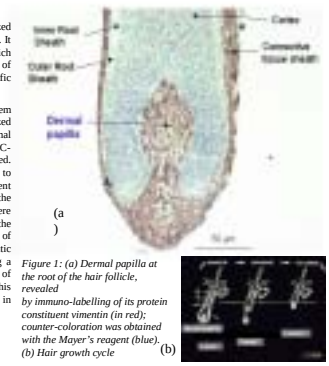

Figure 1: (a) Dermal papilla at the root of the hair follicle, revealed by immuno-labelling of its protein constituent vimentin (in red); counter-coloration was obtained with the Mayer's reagent (blue). (b) Hair growth cycle

RESULTS

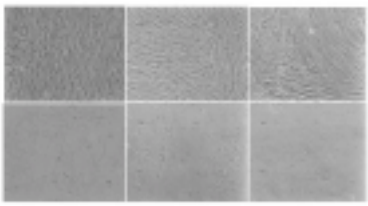

Figure 2: Photographs of the three analysed cultures: before elimination of the cells (a to c, cultures (P13, 10d), (P13, 11d) and (P15, 10d), respectively), and after (d to f).

1) Sample preparation

Three cultures of fibroblasts were used for preparing extracellular matrix (ECM). They differed in the number of passages of the cells (P13 or P15) and in the age of the culture (10 or 11 days). The three obtained protein samples are designated as S(P13, 10d), S(P13, 11d) and S(P15, 10d), respectively. The two first ones were prepared from cultures started on the same day, with a similar initial number of cells. The comparison of their ECM could be expected to give information on the overall repeatability of the analysis. On the opposite, sample S(P15, 10d) was prepared from a culture started separately, with cells having 15 passages instead of 13. The initial numbers of cells seeded in the culture dishes were not counted. **Figure 2** represents the three cell cultures just before fibroblast elimination, and after uncovering the ECM, which appears as a densely striated surface.

Immunofluorescence labelling against laminin, a well characterized component of the ECM, was performed before and after elimination of fibroblasts with the detergent DOC (**Figure 3**). Before fibroblasts elimination, the cell shapes can be recognized, whereas laminin appears as an intricate network after cell disruption and elimination.

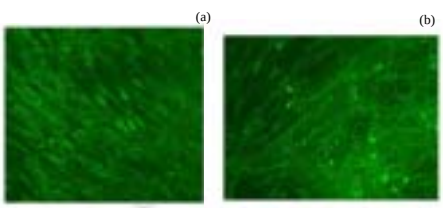

Figure 3: Immunofluorescence labelling of laminin: (a) before elimination of the fibroblasts, (b) after elimination of the cells

2) SDS-PAGE of ECM proteins

More ECM protein material was obtained from the (P13, 10d) and (P13, 11d) cultures than from the (P15, 10d) one. The former cell cultures may be considered as denser (Figure 2a and 2b versus 2c), and may contain a larger amount of ECM than the latter. A difference in growth behaviour may also be expectable between P13 and P15 cells. Comparable volumes of samples were obtained in the three experiments (100-150 µL), but the protein concentration was 0.2 µg/µL with P15 cells, against 0.6 µg/µL for P13 cells. As a result, the protein amounts loaded on the gel wells were also different (**Figure 4**). Gel slices were cut so as to correct for these discrepancies and use similar protein quantities in LC-MS/MS analyses. **For analysing 8 µg of total protein, 2/3 of lane S(P13, 10d), 1 lane of S(P13, 11d) and 2 lanes of S(P15, 10d) were cut.**

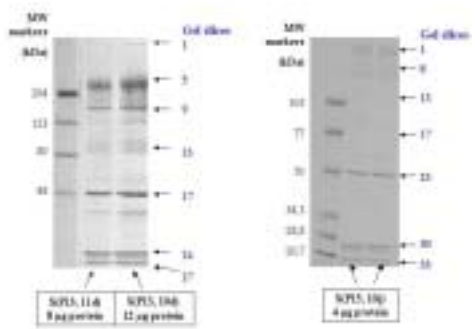

Figure 4: Gels of ECM proteins (stained with Coomassie-blue). The amount of protein charged in each well is indicated.

3) Analysis optimisation

We evaluated the **influence on protein identification results of the maximum number of co-eluted species selected for MS/MS**. The digestion products of 10 gel slices from sample S(P13, 11d) were analysed in two conditions: selection for fragmentation of up to 3 or up to 8 co-eluted ions. The number of identified proteins, interpreted MS/MS spectra and the cumulated Mascot scores of the identified proteins were compared. When pooling the data from the 10 gel slices, the selection of **3 co-eluted precursors for MS/MS fragmentation** appeared to provide better results according to the three previous criteria. Accordingly, all the other analyses of gel slices from the three ECM samples were conducted with this analysis condition.

CONCLUSION

Proteins of the ECM of human dermal fibroblasts were analysed by SDS-PAGE – 1DLC-MS/MS and by 2DLC-MS/MS. The two techniques are likely to provide complementary protein identifications. The analysis of three different cultures highlighted that it is indispensable to precisely control the culture characteristics (number of passages of the cells, number of cells seeded in the culture dishes, number of days of confluence) to obtain reproducible protein samples, and then reproducible protein identifications. Indeed, even if the qualitative and quantitative protein compositions of the three studied ECM samples may be similar, the co-purification of other proteins (soluble, membrane-bound, and from the cytoskeleton) impacts on the LC-MS/MS analysis results, by modifying the peptide population to be analysed by the mass spectrometer. In a further step, we intend to compare the ECM synthesized by dermal fibroblasts and by fibroblasts of dermal papilla. Such a study will require to control as precisely as possible the characteristics of the two cultures.

The optimisation of the salt gradient profiles should aim to obtain a better-balanced distribution of the peptides among the different analysis steps. So the mass spectrometer would have to analyse peptidic mixtures of similar complexity, and eventually provide a better overview of the proteins present in the ECM samples.

Most ECM proteins contain carbohydrate modifications (mainly N-linked). The enzymatic elimination of these carbohydrate moieties might help to obtain a larger protein sequence coverage.

ACKNOWLEDGEMENTS

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MATERIAL AND METHODS

Cultures

Human dermal fibroblasts (F360-cc), either at their 13th or 15th passage (P13 or P15), were cultured in 100-mm diameter dishes until confluence (10 or 11 days of culture). A 10-day culture of P13 cells is designated as (P13, 10d).

Extracellular matrix preparation

Cultures were rinsed 3 times with 5 mL PBS. Cells were eliminated by 3 consecutive treatments with 5 mL of a solution of PBS/water 1:4 (v:v) containing 0.5% sodium deoxycholate (DOC) (w:v). After complete elimination of the cells, the matrix was rinsed twice with PBS. All the used solutions were kept ice-cold.

- For subsequent analysis by SDS-PAGE, the matrix was solubilized by addition of 30 µL of (SDS 5%, Tris 100 mM pH 6,8), heated at 96°C for 5-10 min and centrifuged at 14000 rpm for 15 min (centrifuge 5415C, Eppendorf). The obtained SDS-soluble supernatant is designated as S. After addition of sample buffer (LDS Sample and Sample reducing, Biorad kit), S was heated at 70°C for 10 min, and then separated on a gradient gel (NuPAGE gel 4-12% acrylamide/MOPS running buffer, H = 7 cm, Invitrogen).

Three cultures of fibroblasts were analysed: (P13, 10d), (P13, 11d) and (P15, 10d). Estimation of protein concentration (D_c protein assay, Biorad): 0.6 µg/µL protein in 130-150 µL for (P13, 10d) and (P13, 11d); 0.2 µg/µL for (P15, 10d) in 100 µL.

- For direct 2DLC-MS/MS analysis, the ECM of a (P15, 10d) cell culture was further washed once with water, to eliminate salts from PBS. Then, 500 µL of Tris 100 mM, pH 8.8 containing 5 µg of trypsin (porcine, modified, Roche) were added into the culture dish. Enzymatic digestion was carried out at 37°C for 15 h .

Immunofluorescence labelling

For laminin immunolabelling, the cells or ECM were fixed with MeOH (-20°C, 10 min), labelled with an anti-laminin rabbit antibody (Ab) in the presence of goat serum, and this first Ab was revealed with a FITC-labelled anti-rabbit IgG Ab. For actin and fibronectin dual labelling, the ECM was fixed with PFA (4% in PBS, 10 min, RT); actin was revealed with rhodamin-phalloidin and fibronectin was labelled with a FITC-labelled goat IgG.

Protein digestion

The lanes corresponding to each sample were regularly cut along their whole length (Figure 4). Gel slices were reduced by dithiothreitol (DTT) and alkylated by iodoacetamide (IAA) before tryptic digestion (modified trypsin, Roche).

Proteins pre-digested in the culture dishes were heated at 95°C for 10 min and centrifuged at 14000 rpm for 15 min. The supernatant was reduced with 10 mM DTT for 30 min at 56°C before overnight tryptic digestion at 37°C.

LC-MS/MS analyses

Peptides extracted from each gel slice were analysed by automated 1DLC-MS/MS (Famos-Switchos-UltiMate, LC Packings, Dionex, coupled to a nanoESI-Qtof2, Micromass, Waters). Analytical column: 75 µm d.i. x 150 mm L, C18 PepMap.

Solvents: A= (H_2O,acetonitrile/formic acid, 96/4/0.1, v:v) , B = (H_2O/acetonitrile/formic acid, 10/90/0.085, v:v)

Gradient: 0 => 45% B in 50 min, flush with 100% B for 15 min.

Fragmentation duration in MS/MS: 6 s if peptidic samples came from intense protein bands; 10 s if they were from pale bands.

In 2DLC-MS/MS analyses, the digestion product of ECM proteins was injected onto a SCX-precolumn (Partisil, Whatman, LC Packings, Dionex, 500 µm i.d. x 15 mm L), in series with a RP-precolum (C18 PepMap, LC Packings, Dionex, 300 µm d.i. x 5 mm L). The sample was eluted by fractions from the SCX precolumn onto the RP precolumn by successive 1h30-long salt gradients. Besides the flow-through fraction (0 mM), fractions were eluted by the gradients 0-6, 6-16, 16-54, 54-300 and 300-1000 mM, followed by a 1h30-long flush with 1M salt. Salt gradients were performed with the solvent A used for RPLC separations, and solvent B = 1M NH_4CH_3COO in (water/acetonitrile, 96/4, v:v), pH adjusted to 2.5 with formic acid. Each peptide fraction loaded on the RP precolumn was analysed in the same conditions as in 1DLC-MS/MS, with 10 s fragmentation per ionic species.

Interpretation of LC-MS/MS data

Fragmentation spectra acquired in automated mode were submitted to the research software Mascot. Database searching was performed with the following criteria: (NCBI, taxonomy: mammalian) and (Swissprot, taxonomy: human).

4) Identified proteins

Proteins	Acc Nber (SwissProt)	MW (kDa)	S(P13,11d)	S(P13,10d)	S(P15,10d)
Heparan sulfate	P98160	469	10	23	6
Tenascin-X	P22105	464	3	6	0
Versican	P13611	372.8	0	0	3
Collagen alpha 3(V1)	P12111	321-343	37	32	34
Collagen alpha 1(XII)	Q99715	333	11	8	0
Fibronectin	P02751	262	45	52	50
Tenascin (Hexabrachion)	P24821	241	13	19	19
Laminin gamma-1 chain	*P11047*	*178*	*0*	*1*	*0*
Nidogen-2	*Q14112*	*151*	*0*	*1*	*0*
Collagen alpha 1(I)	P02452	139	5	6	5
Thrombospondin 1	P07996	129	1	2	0
Collagen alpha 2(I)	P08123	129	0	3	*2*
Fibuline-2	P98095	126.5	9	4	17
Collagen alpha 2(V1)	P12110	108	6	6	17
Collagen alpha 1(V1)	P12109	108.5	8	22	6
EMILIN	Q9Y6C2	106.6	0	4	2
Transforming growth factor-beta induced protein IG-H3 (BIGH3)	Q15582	74.5	5	18	14
Vitronectin	P48819/ P04004	*54.3*	*0*	*1*	*1*
EGF-containing fibulin-like extracellular matrix protein 2	O95967	49.4	0	0	2
Dermatopontin	Q07507	*24*	*0*	*1*	*0*

Protéines	Acc Nber (SwissProt)	MM (kDa)	S(P13,11d)	S(P13,10d)	S(P15,10d)
Tissue transglutaminase !!	P21980	77.2	3	7	3
Serine protease HTRA1 (IGF-binding)	*Q92743*	51.3	0	4	11
Collagen-binding protein 1 or 2 (Colligin 1 or 2)	*P29043 or P50454*	*46.3*	*0*	*0*	*2*
Insulin-like growth factor binding protein 5 precursor (IGFBP-5)	*P24593*	*30.3*	*0*	*2*	*0*
Prohibitin	P35232	29.8	0	4	3

Table 2: soluble proteins identified in the three prepared samples of ECM proteins.

Table 1: proteins constitutive of ECM identified in the three prepared samples of ECM.

All the proteins indicated in italic characters in ***Tables 1 and 2*** *were considered as identified in the LC-MS/MS analyses, but their identification was usually based on the fragmentation of one or two peptide(s). Since we can estimate our mass accuracy at roughly 30 ppm, such protein identifications would require a further assay for reliable validation. The number of MS/MS spectra enabling protein identifications are indicated in the last three columns corresponding to the three analysed samples.*

Proteins of ECM

- As a whole, identification of 20 different proteins known to be constitutive of the ECM.

- Nine of them reproducibly identified in the three samples (in blue in Table 1).
- Three proteins only identified in both samples from P13 cells (in red in Table 1).

- Two proteins only detected in the sample provided by P15 cells (in violet in Table 1).

Variations in protein identifications may arise from the fact that:
(i) P13 and P15 cells synthesize ECM at different speeds and in different relative protein compositions,
ii) the number of P13 and P15 cells initially introduced in the dishes may have varied.

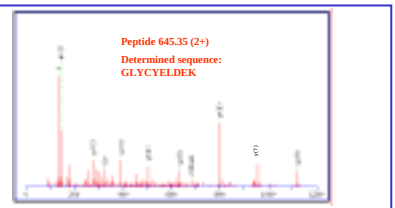

Figure 5: ***The case of vitronectin****: this protein was identified in samples S(P13, 10d) and S(P15, 10d) with one peptide, specific of vitronectin from Sus scrofa (Acc Nber P48819). It differs from the human sequence (Acc Nber P04004) in one residue. Peptide 154-163 of Sus scrofa vitronectin is* ***GLYCYELDEK*** *(****Figure 5****), whereas the homologous human peptide is* ***GQYCYELDEK****. Possible explanations: there may be a mistake in the human sequence, or the studied protein may be a variant of the recorded human vitronectin sequence, not yet listed in SwissProt (release 41.22 of 29th of August 2003). Conclusion: it is recommended to perform database searches in all the mammalian species.*

Other proteins: membrane-bound, soluble, cytoskeletal, contaminants

- As a whole, 16 proteins known to be **attached to the plasma membrane** were identified. This class of proteins involves well-characterized receptors of ECM proteins (**integrins, CD44**). Ten of them were only detected in the LC-MS/MS analyses of sample S(P15, 10d); among these, most were only identified through one unique peptide.
- Five **soluble proteins** were identified, among which 4 ones were only detected in the samples from 10d-cultures (**Table 2**).
- Proteins from the **cytoskeleton** were detected (actins, myosins, tubulins, vimentin, etc). Once again, more numerous proteins of this class were detected in the 10d-samples. **Figure 7** represents the ECM contained in a culture dish after cell elimination, where fibronectin and actin have been immunolabelled; this labelling indicates the presence of residual cytoskeletal filaments in the preparation.
- Finally, some **contaminants** from the nucleus (histones, lamins), from ribosomes, from mitochondria (porins, etc) were also identified. These are highly abundant proteins within the cells.

Significantly more membrane-bound, soluble and cytoskeletal proteins were identified in the 10d-samples. Possible explanation: the cells could be more easily eliminated from the 10d-cultures, than from the 11d-culture, which required a strong manual agitation (the elimination of cells was followed by visual observation under the microscope). So, proteins not strictly constitutive of the ECM (cytoskeletal, soluble and membrane-bound proteins) were more efficiently eliminated from the 11d-sample.

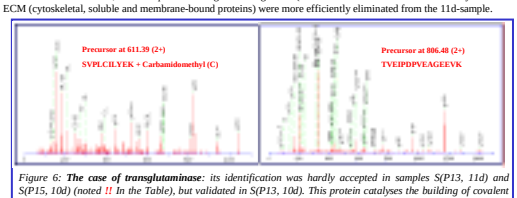

Figure 6: ***The case of transglutaminase****: its identification was hardly accepted in samples S(P13, 11d) and S(P15, 10d) (noted !! in the Table), but validated in S(P13, 10d). This protein catalyses the building of covalent bonds between proteins, which confers to the resulting three-dimensional structure a better resistance to proteolytic degradation. This degradation is performed by matrix metalloproteinases (MMPs), which were not detected in these experiments; yet, the membrane-bound proteins* ***integrin alphaV*** *and protein* ***RECK*** *(Reversion-inducing cysteine-rich protein with Kazal motifs precursor), which interact with the MMPs, were identified in sample S(P15, 10d).*

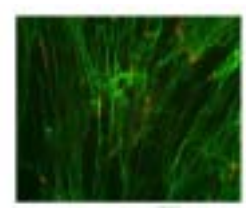

Figure 7: Immunofluorescence labelling of actin (red) and fibronectin (green) after cell elimination

5) Repeatability of protein identifications

The repeatability of protein identifications was assessed on samples S(P13, 10d) and S(P13, 11d), by estimating the influence of the LC-MS/MS analysis step on identification variability. The proteins only detected by LC-MS/MS in sample S(P13,10d) were searched for in sample S(P13,11d). The retention times of peptides leading to their identification were listed. When a protein had been detected in the gel slice G_n of S(P13, 10d), the corresponding peptides were looked for in the gel slices G_{n-1}, G_n, and G_{n+1} of S(P13, 11d). These inquiries were limited to proteins constitutive of the ECM and to soluble proteins.

Protein	N(pept)	Gel slice number	
		S(P13, 10d)	S(P13, 11d)
transglutaminase	5	12	12
serine protease HTRA1	3	15, 16 and 18	/
prohibitin	2	20	19
Insulin-like growth factor binding protein 5	*2*	*25*	*25 (weak)*
dermatopontin	*1*	*23*	*25*
nidogen 2	*1*	*7*	/
laminin	*1*	*7*	/
neprilysin	*1*	*11*	/
vitronectin	*1*	*15*	/

Table 3 : N(pept): number of peptides considered to attempt to find proteins in S(P13, 11d). These peptides were associated to MS/MS spectra providing a reliable identification of the proteins in S(P13, 10d). The two last columns indicate, respectively, the gel slices in which the proteins were detected in S(P13, 10d), and the gel slices of S(P13, 11d) in which they could be detected a posteriori.

Among four proteins detected in S(P13, 10d) with at least 2 peptides, three could be spotted in the MS signal of S(P13, 11d). From a general point of view, when a protein had been identified in S(P13, 10d) through one single peptide, it couldn't be found in S(P13, 11d).

6) Analysis of ECM proteins by 2DLC-MS/MS

Only 1/25 of the digestion product of the ECM of a (P15, 10d) cell culture was analysed per 2DLC-MS/MS run, that is **10 times less protein of S(P15, 10d) than the amount used for SDS-PAGE - LC-MS/MS.** Little material was injected for 2DLC-MS/MS, to be able to perform iterative runs, so as to optimise the analysis conditions (mainly the salt gradient profiles). Preliminary experiments led to the identification of proteins constitutive of the ECM, as well as membrane-bound and soluble proteins; these were mainly proteins previously detected through numerous peptides in SDS-PAGE - 1DLC-MS/MS analyses. Nonetheless, **three proteins not detected in the gel-based approach** could be identified by 2DLC-MS/MS analysis: fibulin 2; the Bone Morphogenic Protein Antagonist 1, gremlin; and a third protein, similar to protein URB/steroid-sensitive protein 1. Each of these proteins was detected through one peptide only.

This preliminary data indicates that 2DLC-MS/MS is likely to provide complementary protein identifications to SDS-PAGE – 1DLC-MS/MS, while reducing the analysis time from around 27 x 1h30 = 40h30 (for the analysis of 27 digested gel slices) to 7 x 1h30 = 10h30 (with 7 salt steps).