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A high molecular arabinogalactan from *Ribes nigrum* L.: influence on cell physiology of human skin fibroblasts and keratinocytes and internalization into cells via endosomal transport

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ABSTRACT

An arabinogalactan protein (F2) was isolated in 1.5% yield from the seeds of *Ribes nigrum* L. (Grossulariaceae) by aqueous extraction and a one-step anion exchange chromatography on DEAE-Sephacel with 24% galactose, 43% arabinose, and 20% xylose as main carbohydrate residues. Methylation analysis revealed the presence of a 1,3-/1,3,6-galactose backbone, side chains from arabinose in different linkages, and terminal xylose residues. The polysaccharide which turned out to be an arabinogalactan protein had a molecular weight of $>10^6$ Da and deaggregated under chaotropic conditions. The cellular dehydrogenase activities (MTT and WST-1 tests) of human skin cells (fibroblasts, keratinocytes) as well as the proliferation rate of keratinocytes (BrdU incorporation ELISA) were significantly stimulated by the polymer at 10 and 100 μg/mL. F2 had no influence on differentiation status of keratinocytes and did not exhibit any cytotoxic potential (LDH test). The biological activity of F2 was not dependent on the high molecular weight. Influence of the polysaccharide on the gene expression of specific growth factors, growth factor receptors, signal proteins and marker proteins for skin cell proliferation, and differentiation by RT-PCR could not be shown. Gene array investigations indicated an increased expression of various genes encoding for catabolic enzymes, DNA repair, extracellular matrix proteins, and signal transduction factors. Removal of terminal arabinose residues by α-L-arabinofuranosidase did not influence the activity toward skin cells, while the treatment with β-D-galactosidase yielded an inactive polysaccharide. The FITC-labeled polysaccharide was incorporated in a time-dependent manner into human fibroblasts (laser scanning microscopy) via endosomal transport. This internalization of the polysaccharide was inhibited by Cytochalasin B.

1. Introduction

The fruits of the black currant (*Ribes nigrum* L., Grossulariaceae) were raised by cultivation from the wild-type red currants, originating from Northern Asia to Europe. The berries contain anthocyanidins, flavonols, pectins, fruit acids, and invert sugar and are commonly used in food technology. During the last years also the seeds of black currants are getting more into the focus for use in cosmetics and dermatology for skin regeneration and neurodermitis. Such a use may be justified because of potential anti-inflammatory and immunostimulating effects of seed extracts,[1,2] especially due to the high content of unsaturated fatty acids (γ-linolenic acid).[3] Additionally seed polysaccharides (arabinogalactan type) from *R. nigrum* were reported to exhibit antiadhesive properties against the adhesion of *Helicobacter pylori* to stomach epithelia.[4] This indicates specific interaction of the polysaccharides from *R. nigrum* with prokaryotic outer membrane adhesins. Recent investigations have shown the presence of arabinogalactan proteins in the seed material of *R. nigrum* which are a family of plant-derived highly glycosylated hydroxyproline-rich glycoproteins, mostly cell-surface proteins, similar to human glycoproteins.[10] The core protein is decorated by arabinose and galactose-rich polysaccharides. AGPs have a strong reactivity with Yariv reagents, synthetical phenylazoglycosides.[8] For review on structure and function of AGP see.[10,36,37] Their amphiphilic nature enables AGPs to be mediators between the cell wall, plasma membrane, and cytoplasm.[37] Beside the specific effects within plant develop-

Abbreviations: AEC, anion exchange chromatography; BrdU, bromodeoxyuridine; FCS, fetal calf serum; FITC, fluoresceinisothiocyanate; GPC, gel permeation chromatography; HaCaT, human adult low calcium high temperature keratinocyte cell line; HPAEC-PAD, high pressure anion exchange chromatography with pulsed-amperometric detection; LDH, lactate dehydrogenase; pNHDFs, primary normal human dermal fibroblasts; pNHEK, primary normal human epidermal keratinocytes; RPS, raw polysaccharide; RT-PCR, real-time polymerase chain reaction.

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ment, physiological effects are described for AGP also on humans, especially concerning a stimulating effect on the innate cellular immune system.[38,39]

The aim of the following study was to investigate potential stimulating effects of the polysaccharides from *R. nigrum* on skin cells under in vitro conditions. Such investigations are related to the finding that certain polysaccharides were found to exhibit activity on skin regeneration and wound healing.[5–7] In this context the structural features of the polysaccharides responsible for the physiological effects had to be investigated. Further the possible mode of actions how the polysaccharides can trigger cells into an activated state was in the focus of interest. Especially the question was to be highlighted if high-molecular polysaccharides can also act inside the cell or if they only interact with the cell outer membrane (Table 1).

2. Results

From the defatted seeds of *R. nigrum* L. a raw polysaccharide (RPS) was isolated by aqueous extraction (yield 1.5%) which was further fractionated by AEC on DEAE-Sephacel® using a step gradient: neutral polysaccharides were eluted with water (31.5%, F1), acidic polymers eluted at 0.1 M sodium phosphate buffer (55.3%, F2), and at 0.25 M sodium phosphate buffer (13.2%, F3).

F2 was characterized with respect to carbohydrate composition, indicating the presence of a type II arabinogalactan with 1,3-/1,3,6-galactose backbone and side chains from arabinose and high amounts of xylose (Table 1).

F2 showed a positive reaction within a radial agarose diffusion test with Yariv reagent,[8] indicating F2 to be an arabinogalactan protein (AGP). A protein content of 0.4% of the polysaccharide was determined according to Ref. 9. The respective amino acid composition, as determined by HPAEC-PAD with glutamine, alanine, glycine, and cystine as the main compounds as shown in Table 2, indicated the presence of a nonclassical low-hydroxyproline/cystine-rich AGP.[10,35]

The molecular weight of F2 turned out to be extraordinarily high, as the polymer eluted within GPC on Sepharose® CL6B (V_o about 1×10^6 Da) and HPLC on Bio-Sil SEC 250 stationary phase in the void volume (Fig. 2). F2 was treated under chaotropic conditions in 8 M urea. Chromatography on Sepharose® CL6B with urea as mobile phase (Fig. 2) resulted in a partial splitting into the resistant polymer (F2*1) and one distinct subfraction (F2*2). This indicates that F2 is a non-covalent aggregate. By atomic absorption spectroscopy the presence of calcium and magnesium ions in F2 was proven. No deaggregation was observed when F2 was dissolved in EDTA and chromatographed via GPC with EDTA 0.1 N as mobile phase. This indicated that the self-aggregation of F2 is not mediated by Ca^{2+}/Mg^{2+} uronic acid–polymer interaction.

Table 1
Carbohydrate composition of F2 from *R. nigrum* seeds as determined by HPAEC-PAD after TFA hydrolysis against external standard calibration and linkage analysis after methylation analysis of carboxy-reduced F2 and GC–MS identification

Carbohydrate	Amount (HPAEC) (mol %)	Linkage type	Amount (GC–MS)
L-Arabinose	42.9	1-(f)	8.6
		1,3-(p)	1.1
		1,5-(f)	20.4
		1,3,5-(f)	1.1
		1,2,5-(f)	11.8
D-Galactose	24.4	1-	0.7
		1,3-	11.5
		1,6-	0.7
		1,3,6-	11.5
D-Xylose	20.1	1-(p)	16.3
		1,3-	2.9
		1,4,6-	1.0
Mannose	4.3	1-	1.4
		1,4-	2.8
Glucose	3.6	1,3-	1.2
		1,4-	2.4
Rhamnose	2.6	1-	2.6
Fucose	1.4	1-	1.4
Galacturonic acid[a]	0.4	1,3-	0.4
Glucuronic acid[a]	0.3	1,4-	0.3

Quantitative PMAA evaluation related to the respective monosaccharide amounts as determined by HPAEC.
[a] Determined as C-6-reduced Gal.

Table 2
Amino acid composition of F2 protein part from *R. nigrum* as determined by HPAEC-PAD on AminoPac™ after hydrolysis with 6 M HCl, 110 °C, 6 h, and tryptophan determined after hydrolysis with 4.25 M NaOH, 110 °C, 20 min

Amino acid	Mol %	Amino acid	Mol %
Arginine	0.0	Isoleucine	0.0
Lysine	1.5	Leucine	2.4
Glutamine	**15.2**	Methionine	3.0
Alanine	**13.2**	Histidine	0.0
Threonine	3.1	Phenylalanine	1.6
Glycine	**12.6**	Glutamate	0.0
Valine	4.8	Aspartate	3.2
Hydroxyproline	4.7	Cystine	**19.8**
Serine	6.1	Tyrosine	0.6
Proline	2.1	Tryptophane	5.6

Biological activities of RPS and the respective fractions on skin cells were investigated using primary normal human fibroblasts (pNHDFs) from human skin and HaCaT keratinocytes, a non-malignant cell line. Both cell types together represent the epidermal and dermal functionality of human skin barrier.

The dehydrogenase activity (MTT- and WST-1 test) and mitogenic cell proliferation rate (BrdU-incorporation ELISA) of cells were determined to evaluate polysaccharide-mediated effects. Potential toxic effects of F2 were determined by the release of lactate dehydrogenase (LDH) as a typical marker for necrosis.

The RPS of *R. nigrum* enhanced the cell viability and proliferation rate significantly in fibroblasts and HaCaT-keratinocytes at 10 and 100 μg/mL (data not shown). AEC fractions F1 and F3 revealed no biological activity (data not shown), while F2 significantly increased cell viability of pNHDF and HaCaT-keratinocytes (Fig. 1). Additionally F2 stimulated the proliferation of keratinocytes, while fibroblasts were not triggered into higher mitogenic status.

Toxicity tests by quantification of LDH-release indicated no signs for cellular damages in the treated groups, indicating F2 to be non-toxic against skin cells.

To clarify if F2 influenced the differentiation behavior of primary normal human epidermal keratinocytes (pNHEK), cells were incubated for 6 days with F2 (10 μg/mL). The early differentiation marker involucrin and the differentiation-specific cytokeratins CK1 and CK10 were monitored by semi-quantitative dot-blot-technique using specific antibodies. The differentiation behavior of NHEK was not affected by F2. At this point of the investigations F2 was assessed to be a strong stimulator of keratinocyte proliferation and cellular dehydrogenase activity.

For some immunostimulating polysaccharides with antitumor effects the activity was shown to be dependent on the occurrence of triple helices.[11,12] In order to clarify whether the stimulating effects of F2 toward skin cells were dependent on the polymer aggregates, F2*1 and F2*2 were isolated on a preparative scale and tested on HaCaT keratinocytes at 10 μg/mL (Fig. 3). The measured effects on cellular dehydrogenase activity for both subfractions were not significantly different to those observed with unfractionated F2.

Treatment of F2 with α-L-arabinofuranosidase resulted in a high molecular weight polysaccharide (F2/1.SF1) with nearly unchanged molecular weight (still in the void volume of GPC