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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

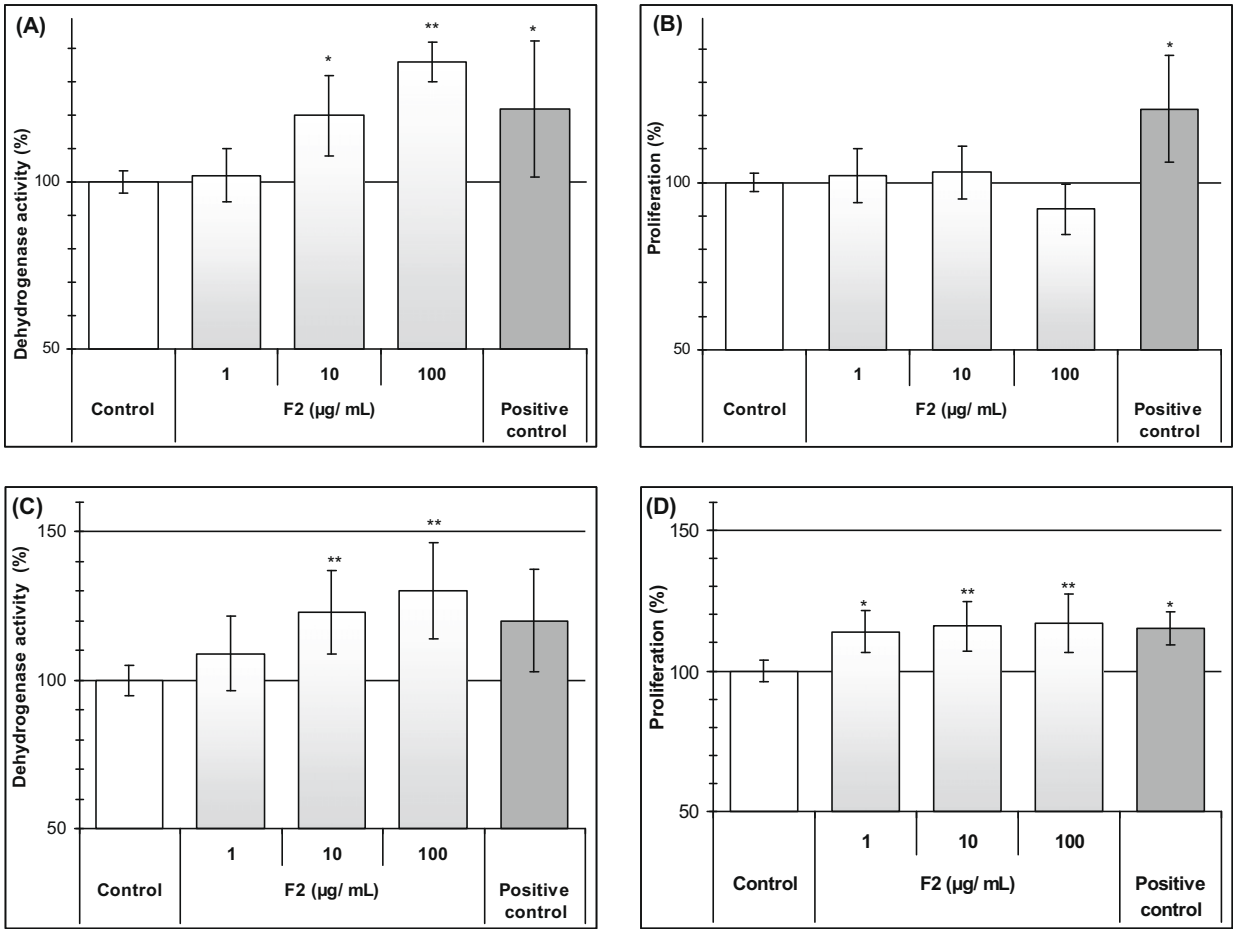

Figure 1. Dehydrogenase activity and proliferation rate of primary normal human fibroblasts (pNHDFs) (A and B) and HaCaT-keratinocytes (C and D) incubated with F2 from *R. nigrum* at 1, 10 and 100 µg/mL over a 48 h incubation period. Bars represent standard deviation (SD) with $n = 6$ replicates from three independent experiments with $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the untreated control group, positive control 1% FCS.

columns), while the arabinose content was reduced by 10% (Table 6). This was in good correlation with the 9% of terminal arabinose residues found during methylation analysis. In parallel xylose content increased from 23% to 32%, indicating that 1-ara residues within the native polysaccharide are directly linked to xylose. The skin cell stimulating activity of F2/1.SF1 was determined by WST-1 test on pNHDF with 132%. This is not significantly different to the effects measured for native F2 (123%) and clearly indicates that the presence of terminal arabinose residues does not contribute to the physiological effects of F2 toward skin cells.

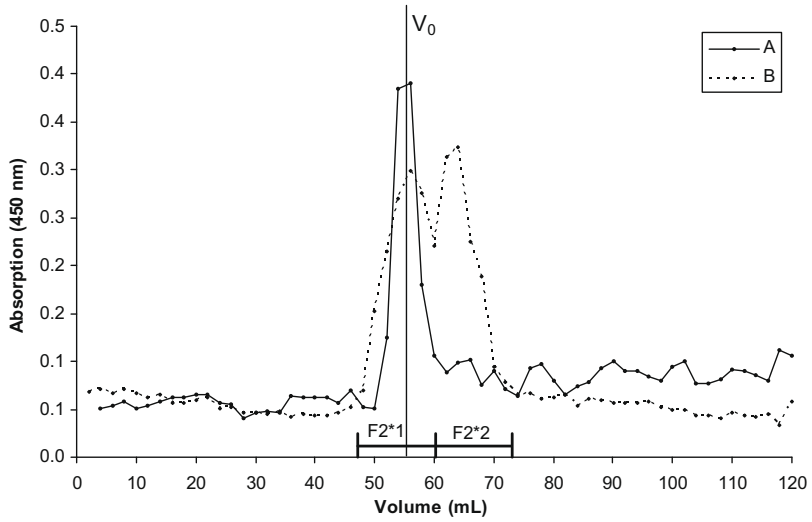

Figure 2. GPC elution profile of F2 from *R. nigrum* on Sepharose®CL6B, eluted with 0.1 N NaCl (curve A), and under chaotropic conditions with 8 M urea (curve B). Eluate obtained with 8 M urea was divided into subfraction F2*1 and F2*2.

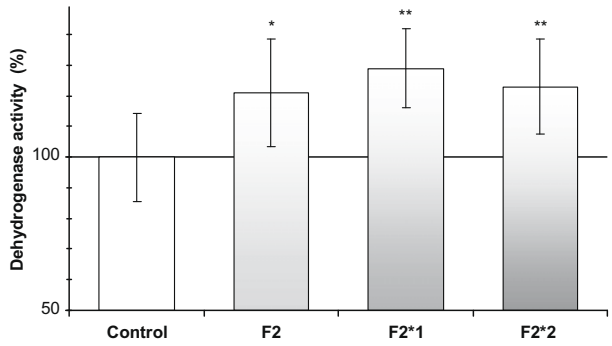

Figure 3. Dehydrogenase activity (MTT test) of HaCaT-keratinocytes incubated with F2 from *R. nigrum* and subfractions F2*1 and F2*2 (deaggregated) obtained after chaotropic treatment of F2 with 8 M urea and GPC separation. Incubation of cells at 10 μg/mL for 48 h. Bars represent SD with $n = 6$ replicates with $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the untreated control group.

Treatment of F2 with β-D-galactosidase resulted in a high molecular weight polysaccharide (F2/4.SF1) with nearly unchanged molecular weight (still in the void volume of GPC columns). The arabinose content of this polysaccharide remained unchanged while the galactose content was reduced from 27% to 22%. In parallel xylose content increased from 23% to 28%, indicating that 1-galactose residues within the native polysaccharide are directly linked to xylose. Concerning the skin cell activity F2/4.SF1 was inactive (105%). This implies that 1-galactose residues are essential for the effects on mitochondrial effects on skin cells.

Other degradation experiments with β-1,3-galactanase failed because of extreme resistance of F2 against this enzyme; also time-dependent partial hydrolysis of F2 with 0.01 M trifluoroacetic acid did not produce defined oligosaccharide fragments in yields sufficient for a further in vitro testing.

The investigation of basic molecular mechanisms of F2 activity was carried out by gene expression studies of growth factors, their receptors, signal molecules, and marker proteins typically described for regulation of proliferation and differentiation of keratinocytes and skin fibroblasts (Table 3). Experiments were performed by quantitative two-step real-time PCR after incubation of the cells with 10 μg/mL F2 over a 24 h period. F2 did not influence any expression of the investigated genes to a significant extent (data not shown). This is in strong contrast to other polysaccharides investigated recently,[5,13] which clearly interact with extramembranous receptors.

To provide an overview about the principle mode of action of F2 gene array studies were initiated, using gene expression analysis using topic-defined PIQOR™ skin microarrays, investigating the expression of 1308 genes related to the acute phase, cytokine signaling, DNA repair, cell metabolism, cell stress and growth, and transcription factors. These genes were investigated from cell lysates from pNHDF after incubation with the test compound. The DNA-sequences were immobilized on a microchip and hybridized with the Cy3- and Cy5-labeled cDNA of the fibroblasts. Level of gene expression of treated and untreated cells was compared by the ratio of fluorescence intensities. Interestingly factors of cell metabolism and DNA repair were upregulated as well as several genes, related to growth factor signal transduction (Table 5). On the other hand, some forms of cytochrome P450 related to metabolism were downregulated as well as TNF-α, TGF-α, and HB-EGF. The enhanced expression of the procollagenase ADAMT S2, syndecan, and collagen and a decreased expression of several matrix metalloproteinases indicate a clear influence of F2 on extracellular matrix proteins.

The data generated by microarray correlated well with the results of RT-PCR. Both methods did not indicate any effect of F2 on any investigated membranous receptors raising the question if F2 acts inside the cell. On the other side this would imply an internalization of the macromolecule into the cell.

For internalization studies F2 was labeled with FITC, purified by GPC on Sepharose® CL6B, and dialyzed. To ensure that fibroblast do not hydrolyze the labeled polymer during the incubation time, cells were incubated for 24 h with 200 μg/mL FITC-F2. The incubation medium was analyzed for the occurrence of unbound FITC by TLC (limit of detection 10 ng FITC). The polymer turned out to be stable under these conditions, no degradation was observed. The cellular uptake of FITC-F2 was analyzed by confocal laser scanning microscopy after labeling of endosomes with Dextran-Texas-Red®,[15] resulting in a red fluorescence and staining of the nucleus with the blue fluorescent dye diaminophenylindol-dihydrochloride (DAPI).[16] A yellow staining after overlay of red and green fluorescence images indicates the localization of FITC-F2 inside the endosomes. After 4 h of incubation no uptake of FITC-F2 into the cells was observed, while the endosomes and the nuclei were stained as expected. After 6 h internalization of the F2 into the fibroblasts and localization inside the endosomes became visible. The fluorescence increased with increased incubation times so that a more intense staining was observed after 14 h (Fig. 4A). These data clearly indicate the uptake of the high molecular polysaccharide F2 into the cells via endocytosis. To prove that unambiguously, fibroblasts were additionally incubated in a follow-up experiment with Cytochalasin B (100 mM), an inhibitor of the subcellular development and movement of endosomes.[17] Laser scanning microscopy indi-

Table 3
Typical growth factors, growth factor receptors, signal proteins and marker proteins from keratinocytes (pNHEK), and dermal fibroblasts (pNHDFs) investigated by RT-PCR on gene expression after treatment of cells with F2; +: expressed

	Functionality	Proliferation	Differentiation	Occurence in pNHDF	Occurence in pNHEK
EGF	Epidermal growth factor	+	+	+	+
EGF-R	EGF receptor	+	+	+	+
KGF	Keratinocyte growth factor	+		+	
KGF-R	KGF receptor	+			+
HGF	Hepatocyte growth factor	+		+	+
Ins-R	Insulin receptor	+		+	+
TGF-ß	Transforming growth factor		+	+	+
FN 1	Marker protein maturation	+		+	
Collagen 1A2	Marker protein maturation	+		+	
Keratin 10	Marker protein differentiation		+		+
Involucrin	Marker protein differentiation		+		+
STAT 6	Signal proteins of IL-4 signal transduction	+		+	+
3PRKC A	Signal protein of Ca^{2+} signal transduction		+		+
PLA2	Signal protein of Ca^{2+} signal transduction		+		+
Smad 3	Signal protein of TGF- β signal transduction		+		+

All factors and proteins are not influenced by treating cells with F2.

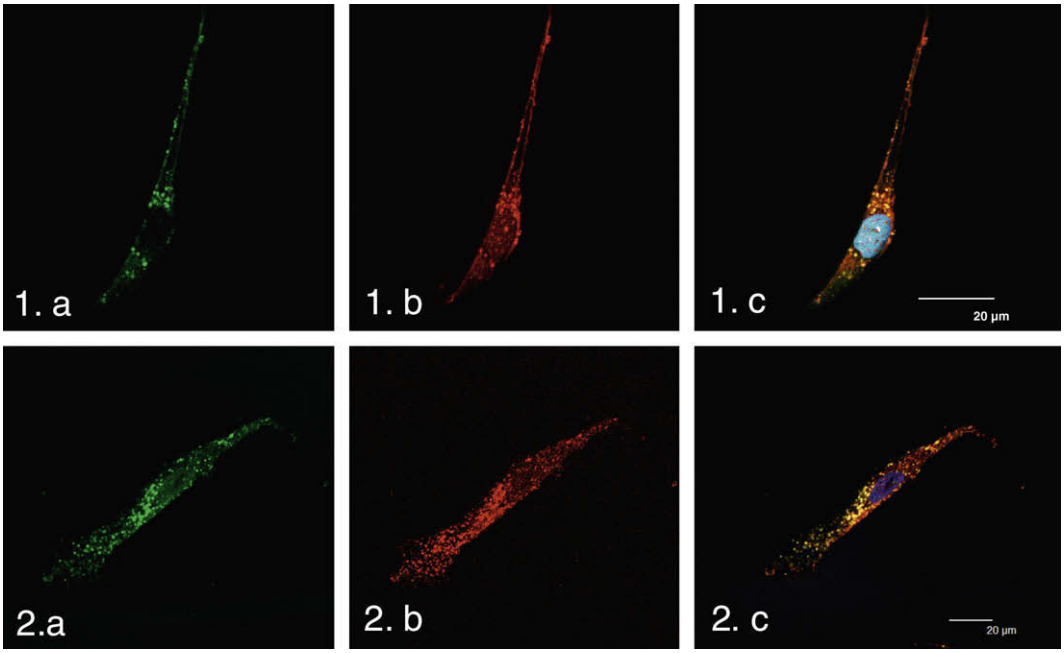

Figure 4. Laser scanning microscopy of cellular localization of FITC-F2 (1.a and 2.a) after 14 h incubation time in primary dermal fibroblasts (pNHDFs). Staining of endosomes with red fluorescent dye Dextran-TexasRed® (1.b and 2.b), nucleus staining with blue fluorescent DAPI. Overlay images (1.c and 2.c) indicate FITC-F2 to be localized within the endosomal structures by the yellow mix color.

cated that no endosomes arose and FIT-F2 was not further internalized any more. From that point of view the endosome-related cellular uptake of the polymer F2 was clearly shown (Table 3).

3. Discussion

In recent literature, the use of black currant seed extracts for various health benefits and especially for dermatological use has mainly been related to the high amounts of unsaturated triglycerides in the fatty oil fraction. The results of the present study indicate that also hydrophilic compounds from seeds are capable of stimulating skin cell activity.

A polysaccharide F2 was isolated from the total water-soluble seed-polysaccharides (RPS), which stimulated dermal fibroblast and keratinocyte cell activity as determined by dehydrogenase activity of both cell types. It was interesting that the proliferation of epidermal keratinocytes was increased significantly, while that of the dermal fibroblasts was not. In case of keratinocytes the enhanced dehydrogenase activity is explainable by the higher proliferation rate resulting in a higher cell number. However in fibroblasts the higher activity maybe a result of an increased number or activity of reducing enzymes as result of an increased metabolism. This shows that the different skin cells have variable responses to the same stimulant. F2 did not exhibit any cell toxicity and did not influence the differentiation status of primary keratinocytes. These effects observed under in vitro conditions are considered to be crucial for new compounds with potential wound-healing activity.[18] The molecular mode of action remains unclear: in most cases known until now an influence of polysaccharides on skin cells is related to interaction with extra-membranous receptors (e.g., EGF-R, KGF-R) or by induction of signal molecules.[5,19] Investigations of the most common signal proteins, receptors and marker proteins in fibroblast, and keratinocyte physiology by real-time-gene expression analysis did not indicate an influence of F2 on the respective genes, as it was determined in former studies for other stimulants of skin cells.[13] A direct interaction of F2 with the receptors, without influencing the receptor- or signal protein expression cannot be excluded. On the other hand, direct activation of EGF-R by an exogenous agonist mostly results in an autokrine upregulation of the respective protein expression, which can easily be monitored by gene expression analysis.[19]

The gene-expression analysis via microarray technology led to a broad information about the influence of F2 on a wide range of physiological parameters in primary human fibroblasts. An increased catabolic activity, as shown in the WST-1 test was also evident on gene expression level. Two intracellular dehydrogenases were upregulated as well as growth factors and factors related to the MAP-kinase pathway. Furthermore F2 enhanced the expression of a variety of factors related to DNA repair, implicating an important impact of F2 on cell regeneration. Moreover downregulation of cytochrome P450 isoforms, responsible for detoxification in cell metabolism, indicates a non-toxic effect of the investigated polysaccharide. However important factors related to tissue development were down-regulated. Examples therefore are members of the TNF superfamily, the fibroblast growth factor-3 or the bone morphogenic protein 10. As expected no parameters of proliferation were upregulated. Additionally F2 seems to have a positive effect on accumulation of extracellular matrix proteins via an enhanced gene expression of the procollagenase ADAMT S2, syndecan, and collagen and a decreased expression of several matrix metalloproteinases.

With respect to the intracellular availability of F2 into fibroblasts a time-delayed uptake over 6–14 h into the cells was shown by laser scanning microscopy. It seems interesting that such high molecular polysaccharides as F2 with MW > 1000 kDa are bioavailable in cells. This indicates that the absorption of polysaccharides into cells and tissue has to be discussed in new way. An internalization via diffusion through the cell membrane was unlikely because of the hydrophilic structure and the high molecular weight of F2. The uptake could also be mediated by specific trans-membrane channel receptors or by endocytosis. The unspecific endocytosis as in case of pinocytosis facilitates internalization of liquids and therein-solute hydrophilic compounds. A specific uptake would be receptor mediated and leads to specific clathrin-coated intracellular vesicles.

The present results indicated that the uptake of F2 into fibroblasts was facilitated by endocytosis whereas the localization inside the same cellular endosomes like the Dextran-TexasRed® suggests an unspecific endosomal internalization. This was considered by a Cytochalasin B treatment resulting in an inhibition of actin filament aggregation and thereby endosome formation.[21] These facts maybe point to an uptake of F2 by actin-driven evaginations described as part of macropinocytosis.[22,23]

Our results suggest that such polymers are indeed capable to influence intracellular biochemical processes directly and not only via binding to membrane receptors, which submit a signal via transduction cascades into the intracellular compartment.

With reference to the structural aspects of F2, the fraction was shown to contain a type II arabinogalactan protein with very low hydroxyproline content untypical for the classical AGP.[10] The coexistence of other high molecular polysaccharides in this fraction cannot be excluded because of the limited separation capacity of the stationary phases within chromatographic clean-up in this very high molecular weight range.

Anyway these types of AGP are found recently in *Daucus carota* cell cultures.[24] The polymer was aggregated from different polysaccharides via H–H-bonds. Deaggregation by chaotropic conditions did not change the biological activity, which indicates that the cell-stimulating activity is not dependent on the high molecular weight as was described for other bioactive polysaccharides, for which the conformation of a triple helix was a prerequisite for activity.[11,12]

The arabinogalactan F2 can be isolated easily and in good yields from *R. nigrum* seeds by simple aqueous extraction and one chromatographic purification step. Therefore and because of the interesting bioactivity, further development toward commercial use of the *R. nigrum* arabinogalactan F2 is very promising and has high potential.

4. Experimental

4.1. General experimentation procedure

If not stated otherwise all chemicals were purchased from Sigma (Deisenhofen, Germany).

R. nigrum L. seeds were obtained from Glaxo Smith Kline Beecham, UK, and were identified by the comparison with reference drug material. A voucher species is deposited in the archives of IPBP, University of Münster 'Johannisbeersamen, BMII/2001'.

4.2. Isolation of RPS and F2

Seed material (174 g) from *Ribes nigrum* L. was crushed under liquid nitrogen to a fine powder. Defatting was performed by Soxlet extraction over 24 h with acetone (yield 159 g). Polysaccharides were extracted three times with 4 L of water at 4 °C under strong stirring. After centrifugation the extracts were combined and the volume was reduced under vacuum (max. 40 °C) and the resulting concentrate (1 L) precipitated in 4 L of ice-cold ethanol 96%. The precipitate was isolated by centrifugation (3000*g*), dissolved in 20 mL water, dialyzed (Cellulose membranes, MWCO 3500 Da, Roth, Karlsruhe, Germany), and lyophilized to yield 1.94%, related to the starting seed material. F2 was isolated by AEC using a DEAE Sephacel® column (30 × 2.5 cm) in the phosphate form and elution by a step gradient of deionized water, sodium phosphate buffers pH 5.0, ionic strengths 0.1-, 0.25-, 0.5-, 1 mol/L, flow 100 mL/h, fraction size 2 mL. Carbohydrate-containing fractions were pooled, concentrated under vacuum, dialyzed, and lyophilized. F2 was isolated from the 0.1 mol/L eluate.

4.3. Carbohydrate analysis

Total carbohydrates in AEC- and GPC-fractions were assayed using the resorcinol–sulfuric acid test.[25] Determination of total uronic acids was performed according to the method of Blumenkrantz[26] with *o*-hydroxydiphenyl in a modification for 96-well-microtiter plates using galacturonic acid as reference. Quantification of monomeric carbohydrates was accomplished on ion-exchange HPLC with pulsed-amperometric detection (Dionex, Idstein, Germany), Bio LC, with AS50 autosampler, GS50 gradient pump, AS50 oven and ED50 electrochemical detector on a CarboPac™ PA1, analytical column, 2 × 250 mm, CarboPac™ PA1, guard column 2 × 50 mm, and BorateTrap™ Trap, 4 × 50 mm. Elution was done with a gradient program using water and NaOH 0.1 M for neutral sugars, and a ternary gradient with water, NaOH 0.1 M and NaOAc 0.5 mM for uronic acids were used.

Polysaccharides were hydrolyzed with trifluoroacetic acid 2 mol/L at 121 °C for 1 h.

Linkage analysis of neutral sugars was analyzed as their partially methylated alditol acetates (PMAA) by GC–MS according to the method of Ref. 27, modified by Ref. 28. GLC was performed on a Agilent 6890N GC–MS system with mass selective detector on a HP-5 MS fused silica capillary column (i.d. 0.25 mm × 30 m, film thickness 0.25 μm) with helium as a carrier gas (pressure: 1.5 bar). Reduction of acidic polysaccharides to the carboxyl-reduced polymers was accomplished in the presence of carbodiimide and $NaBH_4$ following the method of Ref. 29. The determination of molecular weight distribution of polysaccharides was performed by FPLC (GE Healthcare, Freiburg, Germany) on a Sepharose® 6 column and on a low pressure Sepharose® CL6B 100 × 0.8 cm column (GE Healthcare, Freiburg, Germany) using standard dextrans for calibration. Void volume was determined with DextranBlue®. Quantification of residual protein was performed according to the method of Ref. 9 using standard BSA (PAA Laboratories GmbH, Austria) as a reference.

Identity of the D/L forms of xylose, arabinose, and galactose was determined after hydrolysis, derivatization according to the method of Ref. 30 and identification after zone capillary electrophoresis against the respective reference compounds.

4.4. Preparation of FITC-F2[14,15]

F2 (10 mg) was dissolved in 10 mL DMSO. Dibutyltindilaurate (2 μL) and 20 mg FITC was added. The mixture was heated for 2 h at 90 °C. Polysaccharides were precipitated with ethanol (final concentration 80% V/V) and the precipitate dialyzed (MWCO 3500) for 4 days at 4 °C against water. The dialysate was concentrated and purified on Sepharose® CL6B. Fraction eluting in the void volume were pooled, concentrated, and lyophilized; yield 36%.

4.5. Enzymatic treatment of F2

Enzymes used and assay conditions: α-L-arabinofuranosidase from *A. niger*, E.C. 3.2.1.55, Megazyme Ireland, 250 U/mL, assay in sodium acetate buffer, pH 4.2, for 72 h, 40 °C, 2.5 U enzyme.

β-Galactosidase from *A. niger*, E.C. 3.2.1.23, Megazyme, Ireland, 4000 U/mL, assay in sodium acetate buffer, pH 5.0, 72 h, 40 °C, 200 U of enzyme.

β-1,3-Galactosidase, *Xanthomonas manihotis*, recomb. from *E. coli*, E.C. 3.2.1.23, 10 000 U/mL, NEB, U.K., assay in sodium citrate, pH 4.50, 112 h, 37 °C, 100 U of enzyme.

F2, 5 mg, was dissolved in 1 mL of buffer, enzyme solution, and three drops of toluene was added. Incubation was stopped by 3 min, 98 °C, followed by centrifugation (14,000*g*, 10 min) and chromatography of the supernatant on Sepharose®CL6B.

4.6. Methods of cell biology

HaCaT-keratinocytes were kindly provided by Professor Fusenig, Heidelberg. Primary keratinocytes and fibroblasts were obtained from surgical resectates of various caucasian subjects. Approvals of the studies were made by the local ethical committee of University of Münster (2006-117-f-S).

Decontamination of skin and isolation of keratinocytes were carried out according to Ref. 5. For isolation of fibroblasts the dermis was washed with PBS and incubated for 2–3 weeks in cell culture flasks with fibroblast growth medium (MEM high glucose, FCS (10%), and L-glutamine (1%), PAA; Pasching, Austria). Fibroblasts growing out from the tissue formed typical monolayers on the flask, from which cells were isolated and used for further passages.

Permanent culture of HaCaTs was performed in D-MEM high glucose supplemented with FCS (10%), penicillin/streptomycin (1%), glutamine (1%), and non-essential amino acids (1%) (PAA, Pasching, Austria).

Submers cultivation of primary keratinocytes and fibroblasts was performed at 37 °C, 5% CO_2. HaCaT keratinocytes were cultivated at 35 °C, 5%CO_2.

The investigations with pNHEK and pNHDF were carried out with cells of the 2nd to 6th passage.

Before incubation with test polysaccharides cells were directly adapted to serum- and BPE-free Medium (pNHEK: MCDB 153 complete, Biochrom, Berlin, Germany; pNHDF: MEM high glucose, SerEx (10%), and L-glutamin (1%), PAA; Pasching, Austria). In vitro testing of test compounds was performed concerning mitochondrial activity via MTT test,[31] BrdU incorporation assay,[32] differentiation assay,[33] and test on necrosis by extracytosolic lactate dehydrogenase assay.[5] WST-1 test was performed according to the manufacturer's instructions (Roche, Penzberg, Germany). Cultivation conditions, assays, and procedures were according to Refs. 5,13,20,33.

4.7. Gene expression analysis (RT-PCR)

Gene expression studies were performed in principle according to Ref. 33 on pNHEK and pNHDF at a cell density of 5×10^4 cells/mL in 6 well microtiter plates in 2 mL of medium. After 24 h the medium was removed and substituted with PCR medium (pNHDF: MEM high glucose 1% glutamine added, PAA, Pasching, Austria; pNHEK: MCDB 153-medium basal, Biochrom, Berlin, Germany). Incubation with test compounds was done for 24 h. The mRNA was isolated using innuPREP RNA Mini Kit (Analytik Jena, Jena, Deutschland) according to the instructions of manufacturer. RNA content in the samples was calculated in comparison with calibrated agarose gels (1%) after ethidium bromide staining. Reverse transcription was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). cDNA was diluted with RNase-free water to 15 ng cDNA/9 μL. Real-time Polymerase chain reaction (PCR) was done with 1 μL of the TaqMan gene expressions assays (20×) including the primers and probes, Table 4, (Applied Biosystems, Foster City, USA), 10 μL TaqMan

Table 4
Assay IDs analyzed via quantitative real-time PCR

Gene	Assay-ID	Gene	Assay-ID
EGF	HS01099990_m1	KRT10	HS00166289_m1
SMAD3	HS00706299_s1	PLA	HS00179898_m1
PKC-alpha	HS00176973_m1	FN1	HS00415006_m1
18s	HS99999901_s1	Col1A2	HS00164099_m1
FGF7/KGF	HS00384281_m1	EGFR	HS01076068_m1
FGFR2/KGFR	HS00240796_m1	HGF	HS00300159_m1
STAT6	HS00598618_m1	TGF-beta	HS99999918_m1
InsR	HS00169631_m1	IVL	HS00846307_s1

Table 5
Synopsis of genes with changed expression from 1308 genes in total within gene expression analysis using topic-defined PIQOR™ skin microarray from primary normal human dermal fibroblasts (pNHDFs) after a 24 h treatment with F2 (10 μg/mL) compared to an untreated control (= 1)

	Relative gene expression, (SD) (%)
Genes for DNA repair	
NUDT1 (7,8-dihydro-8-oxoguanidine triphosphatase, EC 3.1.6.-)	1.8 (28)
MUTY HOMOLOG (A/G-specific adenine DNA glycosylase, EC 3.2.2.-)	1.7 (34)
XRCC5 (ATP-dependent DNA helicase II, 80 kDa subunit)	3.8 (82)
DDB1 (DNA damage binding protein 1)	1.7 (27)
DDB2 (DNA damage binding protein 2)	1.7 (61)
XRCC9 (DNA repair protein)	2.2 (43)
ERCC-3 (DNA excision repair protein)	2.6 (44)
MLH1 (DNA mismatch repair protein)	2.4 (0)
PAR-1 (proteinase activated receptor 1 precursor)	1.7 (43)
RFC5 (replication factor C 36 kDa subunit)	1.7 (09)
Genes for cell metabolism	
G6PD (glucose-6-phosphate 1-dehydrogenase)	2.2 (71)
MDH1 (malate dehydrogenase, cytoplasmatic, EC 1.1.1.37)	3.7 (86)
MAPK11 (mitogen activated protein kinase 11, EC 2.7.1.37)	1.8 (97)
MEK2 (dual specific mitogen-activated protein kinase kinase 2, EC 2.7.1.)	1.8 (38)
IRF-1 (Interferon regulatory factor 1)	4.6 (95)
IQGAP1 (RAS GTPase activating like protein)	2.0 (96)
MEGF5 (multiple EGF-like domains 5)	3.7 (99)
TNFα (tumor necrosis factor ligand superfamily member 2)	0.4 (69)
IFNG (interferon gamma precursor)	0.4 (27)
TNFRSF10A (tumor necrosis factor receptor superfamily member 10a)	0.4 (46)
TNFSF11 (tumor necrosis factor ligand superfamily member 11)	0.6 (23)
BMP7 (bone morphogenic protein 7 precursor)	0.4 (1)
TGFA (transforming growth factor alpha precursor)	0.5 (43)
HBEGF (heparin-binding EGF-like growth factor precursor)	0.6 (6)
FGF-3 (fibroblast growth factor 3)	0.5 (28)
BMP10 (bone morphogenic protein 10)	0.5 (85)
CYP21A (cytochrome P450 XXIB, EC 1.14.99.10)	0.6 (19)
CYP2B6 (cytochrome P450 2B6, EC 1.14.14.1)	0.4 (79)
CYP2D6 (cytochrome P450 2D6, EC 1.14.14.1)	0.4 (47)
CYP3A5 (cytochrome P450 3A5, EC 1.14.14.1)	0.4 (76)
Genes for extra cellular matrix	
SDC1 (syndecan-1 precursor)	1.8 (59)
SDC4 (syndecan-4 precursor)	1.9 (70)
ADAMTS-2	2.3 (23)
COL8A2 (collagen α 2(VIII))	1.8 (41)
COL6A3 (collagen α 3(VI) chain precursor)	5.4 (105)
MMP12 (matrix metalloproteinase 12 precursor, EC 3.4.24.65)	0.4 (31)
MMP13 (matrix metalloproteinase 13, EC 3.4.24.-)	0.4 (57)
MMP15 (matrix metalloproteinase 15, EC 3.4.24.-)	0.6 (6)

Only relevant data shown. SD: standard deviations from $n = 4$ replicates.

Table 6
Dehydrogenase activity of primary normal human fibroblasts (pNHDFs) incubated with F2 from *R. nigrum*, arabinase- and galactanase-treated *F2* at 10 μg/mL over a 48 h incubation period

Polymer		Ratio Ara-Gal-Xyl (%)			WST-1 activity on pNHDF (%)
		Ara	Gal	Xyl	
F2	Untreated	50	27	23	123 ± 5**
F2/1.SF1	α-L-Arabinofuranosidase, *A. niger*	40	28	32	132 ± 8**
F2/4.SF1	β-D-Galactosidase, *A. niger*	50	22	28	105 ± 8

Bars represent standard deviation (SD) with $n = 6$ replicates with $^*p < 0.05$, $^{**}p < 0.01$ compared to the untreated control group.

Universal MasterMix (2×, without Amperase) (Applied Biosystems, Foster City, USA), and 9 μL of the diluted target cDNA on a 7300 Real-Time PCR (Applied Biosystems, Foster City, USA). For gene IDs of primer sequences see Table 4.

4.8. Gene expression analysis (microarray)

For gene-array analysis pNHDF was cultivated at a cell density of 5×10^5 cells/mL in a cell culture flask (75 cm^2). After 24 h the medium was changed against PCR medium for adaption of cells to minimal culture conditions. Cells were incubated with the test compounds for 24 h in PCR medium.

Cells were harvested after trypsin-treatment, washed with PBS, and frozen in liquid nitrogen. Isolation of the m-RNA and analysis of the gene expression via PIQOR® gene-array microchip technology was performed by Mitenyi Biotech, Köln, Germany. For quality control the RNA integrity number (RIN) was determined with 8.2 (must be >6), indicating sufficient quality for gene expression experiments.[34] For the linear T7-based amplification step 1 μg of total RNA was used and amplified RNA was checked by a Agilent 2100 Bioanalyer. All samples had a Gaussian-like distribution of the transcript lengths as expected (average 0.5–2 kb) (Table 5).

4.9. Internalization experiments of FITC-F2 into fibroblasts

pNHDFs were cultivated on glass slides (Menzel GmbH, Braunschweig, Germany) inside removable silicone chambers (Greiner bio-one, Frickenhausen, Germany) at 2×10^4 cells per chamber in 200 μL growth medium (D-MEM high glucose supplemented with FCS (10%) and L-glutamin (1%); PAA; Pasching, Austria). At 80% confluence cells were incubated with FITC-F2 (200 μg/mL) and 200 μg/mL Dextran-TexasRed® (Invitrogen, Eugene, USA) in growth medium for staining of endosomes for 4, 6, and 14 h. Nucleus staining was done with 1 μg/mL 4′,6-diamino-2-phenyl-indol-dihydrochloride (DAPI) 30 min before end of the total incubation period. Inhibition of endosomal activity was accomplished by incubation with Cytochalasin B, 100 mM for 14 h. After incubation the cells were washed three times with PBS, the silicone chambers were removed, and the object fixed with cover slip and varnish. All procedures were performed by exclusion of direct light. Laser scanning microscopy was performed with a Leica TCS SP2 Fluorescence microscope, Leica, Mannheim, Germany. Excitation λ [nm] 405 DAPI, 488 FITC, 594 TexasRed®, Emission λ [nm] 410-40 DAPI, 500-570 FITC, Texas Red 610-700 (Table 6).

4.10. Statistics

Analysis of the influence of test compounds on cell physiology: statistical evaluation was performed after ANOVA by Dunnett-T post Hoc test. *P*-Values <0.05 were considered significant. All data presented are the means of 12–30 random samples from three independent experiments (errors bars: ±SD).

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