Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

SHORT REPORT

Increase of Dkk-3 Blood Plasma Levels in the Elderly

Christoph Zenzmaier[a], Lilian Sklepos[a] and Peter Berger[a,*]

[a]Endocrinology Division, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria

***Corresponding author**

Peter Berger, Ph.D.
Institute for Biomedical Aging Research
Austrian Academy of Sciences
Rennweg 10
Innsbruck, A-6020
AUSTRIA
Phone: +43-512-583919-24
Fax: +43-512-583919-8
e-mail: peter.berger@oeaw.ac.at

Keywords:

Dickkopf, Blood Plasma, IEMA, Aging

Abbreviations:

BPH, benign prostatic hyperplasia; Dkk, Dickkopf; IEMA, immunoenzymometric assay; FSH, follicle stimulating hormone; HRP, horse radish peroxidase; LH, luteinizing hormone; mAb, monoclonal antibody; NSB, non-specific binding; PCa, prostate carcinoma; SAGE, serial analysis of gene expression.

Abstract

Gene expression of the secreted glycoprotein Dkk-3 is upregulated during cellular senescence in prostate basal epithelial cells and altered in age-related disorders of the human prostate. In order to quantify the influence of such age- and disease-related changes of Dkk-3 levels in body fluids, we established a highly specific and sensitive indirect IEMA. Results revealed a significant increase of Dkk-3 blood plasma levels in the elderly indicating a non negligible physiological role and its use as a marker for senescence not only *in vitro* but also *in vivo*.

Introduction

The secreted glycoprotein Dkk-3 is the most divergent member of the human Dickkopf family (Krupnik et al., 1999; Niehrs, 2006) and in contrast to other family members does not modulate Wnt signaling (Mao et al., 2001; Wu et al., 2000). Our previous results indicated a possible link between Dkk-3 expression and age associated processes in the human prostate. Serial analysis of gene expression (SAGE) of human prostate basal epithelial cells revealed specific induction of *DKK3* gene during cellular senescence (Untergasser et al., 2002) *in vitro*. In a recent immunohistochemical study (Zenzmaier et al., 2008) we demonstrated, that Dkk-3 is downregulated *in vivo* in prostate epithelium of patients suffering from age-related prostate diseases (benign prostatic hyperplasia, BPH and prostate carcinoma, PCa). This downregulation is counterbalanced by increased protein expression in the endothelial cells of the blood vessels supplying the diseased tissue.

To investigate if the identified cellular senescence-related changes in expression became manifest in body fluids, particularly in human blood plasma, we developed a sensitive indirect immunoenzymometric assay (IEMA).

Materials and Methods

Generation and characterization of monoclonal antibodies

A series of mouse monoclonal antibodies (mAbs) against Dkk-3 was raised according to methods published for mAbs against FSH (Berger et al., 1988). To produce recombinant protein for the immunization, the *DKK3* ORF was cloned in-frame upstream of the E epitope tag (GAPVPYPDPLEPR) into the expression vector pcDNA3.1 (Invitrogen). COS7 cells were transiently transfected with the construct

and the protein was purified from the conditioned media via affinity chromatography using a column packed with Anti-E Tag Sepharose (GE Healthcare).

Radioimmunoassays

Antibodies were characterized according to affinity and specificity with native (untagged) Dkk-3 that was purified via HPLC (anion exchange followed by size exclusion chromatography) from conditioned media of stably transfected LNCaP cells (Zenzmaier et al., 2008) and the commercially available homologous proteins Dkk-1, Dkk-4 and Soggy-1 (R&D systems). The proteins were radiolabelled with ^{125}I using chloramine T. Antibodies were incubated over night at 4°C with radiolabelled protein (approximately 25 000 cpm in 100 µL 0.3% BSA in PBS). Separation of bound from free tracer was achieved by 2 h incubation at room temperature on a shaker with 100 µL immunoabsorbent (donkey anti-mouse IgG or donkey anti-goat IgG; Guildhay, respectively, coupled to bromide-activated Sepharose CL-4B beads; GE Healthcare). After 3 times washing with 0.5% Tween-80 in PBS bound radioactivity was determined in 1470 WIZARD automatic gamma counter (Wallac).

Sensitive IEMA protocol

An indirect IEMA was established using mAb INN(sbruck)-Dkk3-1 at a concentration of 4 µg/mL in coating buffer (200 mM $NaHCO_3$; pH 9.5; incubation for 1 h at 37°C) to coat 96-well plates. After a 30 min blocking step with 1% BSA/PBS, wells were incubated with antigen diluted in 1% BSA/PBS over night at 4°C. After 5 times washing with washing buffer (PBS containing 0.05% Tween20 and 0.01% Thiomersal) plates were incubated with the biotinylated polyclonal anti Dkk-3 antibody (R & D Systems) at a concentration of 200 ng/mL in 1% BSA/PBS for 2 h at room temperature. Signals were recorded after incubation with streptavidin / horse

radish peroxidase (HRP; DAKO; 1:500 in 1% BSA/PBS) and the substrate tetramethylbenzidine / H_2O_2 (Substrate Reagent Pack, R & D Systems), each for 30 min at room temperature, by measuring the absorbance at 450 nm (Victor^2 1420 multilabel counter, Wallac). All samples were run in duplicate and results were calculated from specific mean signal (mean – zero standard). Native i.e. untagged Dkk-3 that was purified via HPLC (anion exchange followed by size exclusion chromatography) from conditioned media of stably transfected LNCaP cells (Zenzmaier et al., 2008) was used as a standard. The concentration of the standard was quantified via the absorbance at 280 nm in a HITACHI U-2000 spectrophotometer using the extinction coefficient of 19940 calculated with the ProtParam tool (www.expasy.ch/tools/protparam.html).

Probands

Blood plasma samples were obtained from 63 healthy donors. The local ethical committee approved the bleeding protocol and all participants provided informed consent. A medical history was obtained and individuals with malignancies, acute diseases or advanced stages of severe chronic diseases were excluded from the study. Persons who were under immunosuppressive therapy were also excluded as well as persons under medication for type II diabetes.

Results

A series of mouse monoclonal antibodies (mAbs) against Dkk-3 was raised. Six of these antibodies and an affinity purified goat polyclonal antibody (R & D Systems) were then characterized according to affinity and specificity with recombinant Dkk-3 and the homologous proteins Dkk-1, Dkk-4 and Soggy-1 (R&D systems). None of the tested antibodies had significant affinities towards the homologous proteins. The

antibodies wih the highest affinity towards Dkk-3 (mAb INN(sbruck)-Dkk3-1 and the goat polyclonal antibody) were then used to establish a sensitive IEMA protocol. To quantify the signals obtained in the IEMA recombinant untagged Dkk-3 was used as a standard.

Crossreactivities in the IEMA towards the homologous proteins Dkk-1, Dkk-4 and Soggy-1 (R&D systems) were determined to be << 0.1%. The recovery was calculated by spiking with recombinant protein and was approximately 92%. Assay sensitivity was defined as the mean signal of non-specific binding (NSB = zero standard) plus three standard deviations (NSB + 3SD) and was about 1 pM. The intra- and inter-assay variances (CV%) at a protein concentration of 40 pM were 11% and 13%, respectively. A typical standard curve is shown in Figure 1.

The standard was compared to commercially available recombinant Dkk-3 (R&D systems). Although the commercially available protein has a 10X histidine tag at the C-terminus and was expressed in the mouse myeloma cell line NS0, it yielded signals in the IEMA in approximately the same range as the native (untagged) recombinant protein isolated from a human cell line (data not shown).

Given the specific induction of Dkk-3 during *in vitro* cellular senescence of human prostate basal epithelial cells (Untergasser et al., 2002), protein expression during aging was analyzed *in vivo*. Blood plasma samples from 63 healthy individuals from two age cohorts were investigated (for proband characteristics see Table 1). A mean concentration of 1.42 ± 0.56 nM Dkk-3 (n = 63) was determined, corresponding to 51.3 ± 20.3 ng/mL of the non-glycosylated protein. There were no significant sex-specific differences in the obtained protein levels (female: n=33, mean±SD: 1.42 ± 0.62 nM; male: n=30, mean±SD: 1.41 ± 0.49). When the data were analyzed according to probands´ ages, a significant increase (37.6%; $p < 0.01$; Figure 2A) of the mean blood Dkk-3 levels was observed. The age cohorts were further subdivided

and the age-related increase of the mean plasma protein concentration was confirmed for both sexes (female: 32.5%, $p = 0.03$; male: 44.2%, $p < 0.01$; Figure 2B). The mean values obtained from the single groups are listed in Table 1, a scatter plot of all individual values is depicted in Figure 2C.

Discussion

To our knowledge this is the first quantitative determination of secreted Dkk-3 protein levels. The age-correlated increase of Dkk-3 confirmed our previous *in vitro* cellular senescence data and indicate a non negligible physiological role and its use as a marker for senescence. However, the precise physiological function of the non-gender-specific constitutive presence of high amounts of Dkk-3 in the blood plasma of young and elderly individuals (1.17 ± 0.36 nM and 1.61 ± 0.61 nM corresponding to 42.2 ± 13.0 ng/mL and 58.1 ± 22.1 ng/mL of the non-glycosylated protein) remains to be clarified. Its levels e.g. are significantly higher than those of other important glycoprotein hormones like FSH (physiological range 0.18 – 21.3 ng/mL; Madersbacher et al.,1993) or LH (physiological range 0 – 3.23 ng/mL) in young and elderly probands.

Despite its homology to the Wnt antagonist Dkk-1, Dkk-3 does not modulate Wnt signaling (Mao et al., 2001; Wu et al., 2000). Due to its downreguation in a number of cancer cells (Hsieh et al., 2004; Kurose et al., 2004; Tsuji et al., 2000; Tsuji et al., 2001) Dkk-3 was considered to function as a tumor suppressor but when studying the effect of Dkk-3 on cell growth of either primary prostate cells or BPH and PCa cell lines it appeared that proliferation rates were neither impaired by overexpression nor addition of recombinant protein (Zenzmaier et al., 2008). Thus the biological role of the protein remains unclear.

The origin of the Dkk-3 in blood plasma is not resolved either. One source could be

endothelial cells, were Dkk-3 is reported to be expressed (Goodwin et al., 2006; Kupatt et al., 2005). Moreover, we detected high expression of Dkk-3 in vascular endothelial cells in BPH and PCa tissue sections (Zenzmaier et al., 2008). Upregulation of the protein in tumor endothelium has also been demonstrated in colorectal carcinoma, glioma, high-grade NHL and melanoma (St Croix et al., 2000; Untergasser et al., 2008). Therefore Dkk-3 can be considered a pro-angiogenic protein in neovascularisation. High expression of the protein was also seen in a subset of adult human pancreatic beta cells (Hermann et al., 2007). These cells could also contribute to the Dkk-3 levels determined in human blood plasma.

The established assay may help to elucidate the mechanism by which the protein acts, as it gives us the opportunity to quantify differences in Dkk-3 secretion in *in vivo* and *in vitro* models of aging and disease.

Acknowledgments

The authors wish to thank Michael Keller and Katharina Gapp for their excellent technical support.

References

Berger, P., Panmoung, W., Khaschabi, D., Mayregger, B., Wick, G., 1988. Antigenic features of human follicle stimulating hormone delineated by monoclonal antibodies and construction of an immunoradiomometric assay. Endocrinology 123, 2351-9.

Goodwin, A.M., Sullivan, K.M., D'Amore, P.A., 2006. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. Dev Dyn 235, 3110-20.

Hermann, M., Pirkebner, D., Draxl, A., Berger, P., Untergasser, G., Margreiter, R., Hengster, P., 2007. Dickkopf-3 is expressed in a subset of adult human pancreatic beta cells. Histochem Cell Biol 127, 513-21.

Hsieh, S.Y., Hsieh, P.S., Chiu, C.T., Chen, W.Y., 2004. Dickkopf-3/REIC functions as a suppressor gene of tumor growth. Oncogene 23, 9183-9.

Krupnik, V.E., Sharp, J.D., Jiang, C., Robison, K., Chickering, T.W., Amaravadi, L., Brown, D.E., Guyot, D., Mays, G., Leiby, K., Chang, B., Duong, T., Goodearl, A.D., Gearing, D.P., Sokol, S.Y., McCarthy, S.A., 1999. Functional and structural diversity of the human Dickkopf gene family. Gene 238, 301-13.

Kupatt, C., Horstkotte, J., Vlastos, G.A., Pfosser, A., Lebherz, C., Semisch, M., Thalgott, M., Buttner, K., Browarzyk, C., Mages, J., Hoffmann, R., Deten, A., Lamparter, M., Muller, F., Beck, H., Buning, H., Boekstegers, P., Hatzopoulos, A.K., 2005. Embryonic endothelial progenitor cells expressing a broad range of proangiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia. Faseb J 19, 1576-8.

Kurose, K., Sakaguchi, M., Nasu, Y., Ebara, S., Kaku, H., Kariyama, R., Arao, Y., Miyazaki, M., Tsushima, T., Namba, M., Kumon, H., Huh, N.H., 2004. Decreased expression of REIC/Dkk-3 in human renal clear cell carcinoma. J Urol 171, 1314-8.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., Niehrs, C., 2001. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411, 321-5.

Niehrs, C., 2006. Function and biological roles of the Dickkopf family of Wnt modulators. Oncogene 25, 7469-81.

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K.E., Montgomery, E., Lal, A., Riggins, G.J., Lengauer, C., Vogelstein, B., Kinzler, K.W., 2000. Genes expressed in human tumor endothelium. Science 289, 1197-202.

Tsuji, T., Miyazaki, M., Sakaguchi, M., Inoue, Y., Namba, M., 2000. A REIC gene shows down-regulation in human immortalized cells and human tumor-derived cell lines. Biochem Biophys Res Commun 268, 20-4.

Tsuji, T., Nozaki, I., Miyazaki, M., Sakaguchi, M., Pu, H., Hamazaki, Y., Iijima, O., Namba, M., 2001. Antiproliferative activity of REIC/Dkk-3 and its significant down-regulation in non-small-cell lung carcinomas. Biochem Biophys Res Commun 289, 257-63.

Untergasser, G., Koch, H.B., Menssen, A., Hermeking, H., 2002. Characterization of epithelial senescence by serial analysis of gene expression: identification of genes potentially involved in prostate cancer. Cancer Res 62, 6255-62.

Untergasser, G., Steurer, M., Zimmermann, M., Hermann, M., Kern, J., Amberger, A., Gastl, G., Gunsilius, E., 2008. The Dickkopf-homolog 3 is expressed in tumor endothelial cells and supports capillary formation. Int J Cancer 122, 1539-47.

Wu, W., Glinka, A., Delius, H., Niehrs, C., 2000. Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. Curr Biol 10, 1611-4.

Zenzmaier, C., Untergasser, G., Hermann, M., Dirnhofer, S., Sampson, N., Berger, P., 2008. Dysregulation of Dkk-3 expression in benign and malignant prostatic tissue. Prostate 68, 540-7.

Figure Legends

Figure 1. Typical standard curve for the Dkk-3 IEMA. Specific signals (signal – non-specific binding (NSB; zero standard) from doubling dilutions of recombinant protein from 140 pM to 0.5 pM are depicted. The sensitivity limit of the assay (specific signal > three times the standard deviation of NSB) is approximately 1 pM. Unknown samples were diluted in ELISA buffer to Dkk-3 concentrations between 10 and 100 pM.

Figure 2. Age-correlated increase of blood plasma Dkk-3 levels. Dkk-3 concentration in blood plasma was determined from 63 individuals (33 females / 30 males). The blood Dkk-3 content is significantly elevated in elderly persons (A; young: n=27, old n=36) of both genders (B). A scatter plot of all values with linear regression curves for the gender cohorts demonstrates, that the slope of the age-correlated increase is similar for females and males (C).

Table(s)

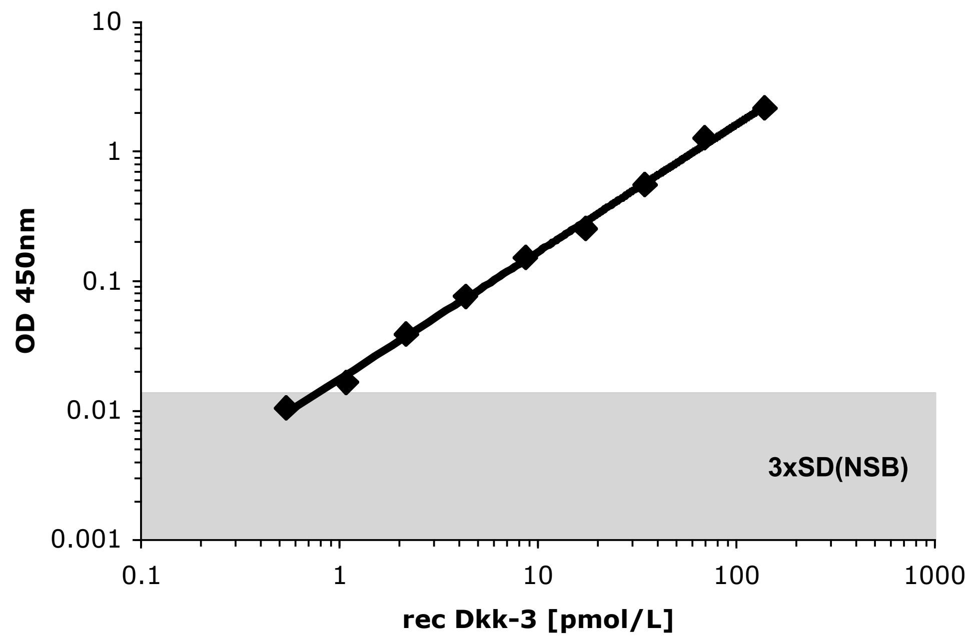
Table 1: Study participant age characteristics and Dkk-3 ELISA results

	All Individuals		Female		Male	
	Young	Old	Young	Old	Young	Old
Number of individuals	27	36	14	19	13	17
Age (years)						
mean±SD	25.1±3.7	73.0±7.1	23.4±3.6	70.9±7.0	27.0±2.8	75.4±6.6
Dkk-3 (nmol/L)						
mean±SD	1.17±0.36	1.61±0.61	1.20±0.31	1.59±0.74	1.13±0.42	1.63±0.44
p	< 0.01*		0.03**		<0.01**	
Median	1.09	1.54	1.16	1.38	0.96	1.68
Range	0.49-1.79	0.57-3.42	0.79-1.76	0.57-3.42	0.49-1.79	0.95-2.66

SD: standard deviation

p values are calculated as two-(*) or one-sided(**) student´s t-test

Figure(s)



Figure(s)