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The Immunomodulatory Activity of Peptides Related to the DNA Contacting Loop of p53 Protein

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Taking into account the sequence homology existing between thymopoietin II and the DNA-binding domain of p53 protein, a series of octapeptides was synthesized, related to the wild p53 type protein as well as to its mutated forms, appearing in some human tumours. The wild type octapeptide has immunostimulative activity with regard to the humoral immune response, but is inactive in the cellular immune response. The mutated peptides of p53 differ in their immunomodulatory activity from the wild type octapeptide. The Ser5 analogue of the wild type peptide is a strong stimulant of the humoral immune response and enhances TNF-α production, while at the same time suppressing the cellular immune response. The data suggest that the mutations of p53, which favour tumour development and growth, may also change the immune activity of respective p53 fragments.

Keywords: p53 protein; peptide immunomodulators; TP5 analogues

INTRODUCTION

The human p53 protein, a tumour-suppressor gene product, controls the cell cycle, inducing growth arrest during the C_1 phase and probably plays an important role in programmed cell death [1]. The mutated forms of p53 were found in many human tumours [2]. Mutations may lead to tumour cell growth because the mutated forms of p53 lack the regulatory activity of the p53 wild type protein.

Recently, the three-dimensional structure of p53 was solved by X-ray analysis [3]. The protein molecule is composed of an N-terminal transactivation domain, an oligomerization domain, a central DNA-binding domain and a C-terminal regulatory domain. The majority of point mutations, leading to tumour growth, appear in the 245–249 fragment of the DNA-binding domain [4]:

$$\underset{245}{-Gly-Met-Asn-Arg-Arg-}\underset{249}{Pro-}$$

The Arg^{248} residue in this sequence plays a crucial role in the p53 - DNA binding [3].

The $Gly^{245} \rightarrow Ala^{245}$ mutation is specific for some bladder cancers [5], $Met^{246} \rightarrow Ile^{246}$ mutation for the lung cancers [6], and $Arg^{249} \rightarrow Ser^{249}$ mutation for liver [7–9], oesophageal [10], breast [11] and lung [12] cancers.

In the past few years we started a search program for new peptide immunomodulators, looking for thymopentin-like (TP5-like) structures within the sequences of important immunoregulatory and defence proteins. Thymopentin is a pentapeptide with the sequence: Arg-Lys-Asp-Val-Tyr. It is an active fragment of a thymus polypeptide, thymopoietin II, a very well-known immune system stimulant. Using this approach we demonstrated the presence of an immunosuppressive mini-domain in the lactoferrin molecule [13], and also showed the immunomodulatory diversity of proteins belonging to the transforming growth factor beta (TFGβ) family [14]. During these investigations we found that the substitution of Asp residue of TP5-like peptides by Pro leads very often to peptide immunosuppressors (e.g. Arg-Lys-

Abbreviations: PFC, plaque-forming cell; DTH, delayed type hypersensitivity; TNF-α, tumour necrosis factor α; IL-1, IL-2, IL- 6, interleukin-1, -2, -6, respectively; TGF-β, transforming growth factor β; SRBC, sheep red blood cells; TP5, thymopentin.

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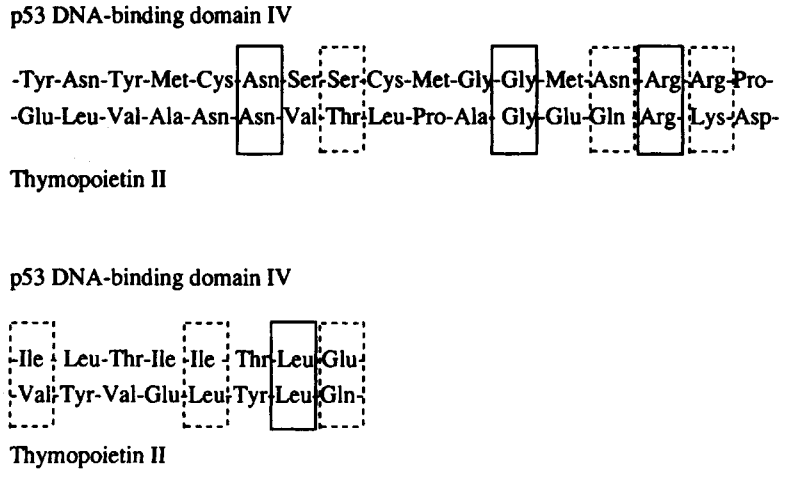

Scheme 1

Pro-Val-Asp – immunosuppressive fragment of lactoferrin; and Arg-Lys-Pro-Lys-Val – immunosuppressive fragment of TGFβ_1). Also in the p53 molecule there is a similar TP5-like sequence located within the DNA-binding domain of the protein namely Arg-Arg-Pro-Ile-Leu – 248–252 fragment of p53.

The comparison of the sequence of the DNA-binding domain of human wild type p53 protein [15] with that of the bovine thymopoietin II [16] shows a distinct homology between those polypeptides as shown in Scheme 1.

Taking into account the identical positions of Asn, Gly, Arg and Leu residues in both polypeptides (indicated by solid line boxes), and the same positions of the pairs of similar amino acids (Ser-Thr, Asn-Gln, Arg-Lys, Ile-Val, Ile-Leu, and Glu-Gln; indicated above by broken line boxes), the homology between the peptides may be established as equal to 40%. For human thymopoietin [17] the homology is somewhat less (32%). However, if in the last case the identically located dipeptide fragments Met-Asn (in p53) and Glu-Met (in thymopoietin) are considered to be homological, the homology increases to 40%.

Taking these data into account, the fragment of p53 wild type protein, Gly-Met-Asn-Arg-Arg-Pro-Ile-Leu was synthesized together with several mutated analogues as shown in Table 1.

The aim of this work was to answer the questions: (i) Does the indicated fragment of p53 possess any immunomodulatory potency? (ii) Are the immunomodulatory properties of this fragment changed if the mutated p53 proteins are taken into account? (iii) Are the changes cumulated if two respective mutations are present in the octapeptide?

MATERIALS AND METHODS

Peptides

All the peptides were synthesized by the t-Boc solid-phase strategy, on a Merrifield resin (Biorad, 0.7 mmol/g). C-terminal residues were attached to the resin via the caesium salt method. The side chains of arginine and methionine were protected by nitro- and sulfoxyl- groups respectively. The serine-containing peptides were synthesized from the C-terminus through the residues preceding serine, with DCC as coupling reagent. The remaining residues (serine through N-terminal) were incorporated with BOP. The peptides that did not contain serine were synthesized by standard DCC strategy. The cleavage of the peptides was performed by 'high TFMSA' or 'low– high TFMSA' procedures. The 'low–high TFMSA' was applied to the peptides containing methionine residues. The products were precipitated with diethyl ether and lyophilized from water. The crude peptides were subsequently purified by reversed-phase HPLC. The trifluoroacetate counterions were exchanged to acetates by ion exchange chromatography. The purity and homogeneity of each peptide (in all cases better than 95%) was confirmed by RP-HPLC, amino acid analysis and FAB mass spectrometry. The analytical data for all the peptides is summarized in Table 1.

Biology

The influence of peptides on both the humoral and the cellular immune responses was measured. The peptide activity on the humoral immune response

Table 1 Analytical Data of p53 Protein-related Peptides

Peptide	Yield (%)[c]	MW calc./found	R_t[a] (min)	Amino acid analysis
GMNRRPIL	80	956/956	23.94	$G_{0.93}M_{0.96}N_{0.95}{}^{b}R_{2.06}P_{1.02}I_{0.99}L_{1.01}$
AMNRRPIL	80	970/970	23.10	$A_{0.95}M_{0.96}N_{0.95}R_{2.20}P_{1.20}I_{0.99}L_{1.00}$
GINRRPIL	50	938/938	23.20	$G_{1.00}I_{1.98}N_{0.95}R_{2.10}P_{1.20}L_{1.00}$
GMNRSPIL	50	887/887	21.01	$G_{0.95}M_{0.95}N_{0.98}R_{1.01}S_{0.95}P_{1.00}I_{0.95}L_{0.98}$
GINRSPIL	30	869/869	21.30	$G_{1.00}I_{1.98}N_{0.95}R_{1.20}S_{0.99}P_{1.10}L_{1.01}$

[a] Retention time (min) in analytical RP-HPLC. Concentration 1 mg/ml. Gradient 0– 100% A/60 min, A-80% acetonitrile + 0.1% TFA in water, B-0.1% TFA in water; column 250 × 4.6 mm RP-C18 ODS (Beckman), flow rate 1 ml/min.
[b] Determined as aspartic acid.
[c] Peptide content in a crude product determined by RP-HPLC.

Table 2 PFC Number in CBA/Iiw Mouse Spleen Cell Cultures Immunized with SRBC and Treated with Peptides Investigated

Peptide	Dose (μg/ml)	PFC/10^6	± SE	*P* Student test	%[a] of stimulation
Control 0.9% NaCl	–	845	52		
GMNRRPIL	1	880	176	NS	
	10	1537	230	< 0.02	82
	100	1519	338	< 0.02	80
AMNRRPIL	1	1426	148	< 0.05	69
	10	1903	145	< 0.01	125
	100	1984	131	< 0.01	135
Control 0.9% NaCl	865	16			
GINRRPIL	1	640	74	NS	
	10	885	57	NS	
	100	1087	144	NS	
Control 0.9% NaCl		845	52		
GMNRSPIL	1	1455	52	< 0.05	72
	10	2663	219	< 0.001	215
	100	7900	324	< 0.001	835
GINRSPIL	1	2407	317	< 0.001	185
	10	2528	313	< 0.001	199
	100	2442	139	< 0.001	189

[a] % of stimulation = $100\left(\frac{\text{experimental value}}{\text{control}} - 1\right)$.

was determined by the Jerne plaque-forming cell test as modified by Mishell and Dutton [18]. The cellular immune response was measured in the food pad by assessing the delayed type hypersensitivity reaction. DTH was measured according to Lagrange *et al.* [19]. Details of both tests were described recently [20].

CBA/Iiw and 129/Iiw mice (8–10 weeks old) were used for the PFC and DTH tests respectively. Groups of five mice in every PFC experiment and seven mice in every DTH experiment were used. In the case of PFC *in vitro* experiments the results were expressed as mean ± SE of six wells. The results, expressed as PFC numbers and DTH units, were statistically elaborated by Student *t*-test.

Influences on the production of interleukin-1 (IL-1) interleukin-6 (IL-6) and tumour necrosis factor

Table 3 PFC Number in the Spleen Cells of CBA/Iiw Mice Immunized with SRBC and Treated i.p. with the Peptides Investigated − 3 h and +24 h after Antigen Administration

Peptide	Dose (μg/mouse)	PFC/10^6	±SE	*P* Student test	%[a] of stimulation
Control 0.9% NaCl	–	1683	36		
GMNRRPIL	10	2229	252	<0.05	32
	100	2236	143	<0.05	33
AMNRRPIL	10	1640	187	NS	
	100	2876	223	<0.01	71
Control 0.9% NaCl		1185	61		
GINRRPIL	10	1693	197	<0.05	43
	100	1676	183	<0.05	41
Control 0.9% NaCl		1683	36		
GMNRSPIL	10	1747	102	NS	
	100	2382	111	<0.01	42
GINRSPIL	10	2590	142	<0.01	54
	100	3028	159	<0.001	80

[a] % of stimulation = $100\left(\frac{\text{experimental value}}{\text{control}} - 1\right)$.

alpha (TNF-α) were assessed using the P-388-DI cell line. For interleukin-2 (IL-2) production the Jurkat E6.1 cell line was used. Details of procedures are described elsewhere [21].

RESULTS

Table 2 shows that the peptides Gly-Met-Asn-Arg-Arg-Pro-Ile-Leu from the DNA-binding loop of p53 wild type protein and Ala-Met-Asn-Arg-Arg-Pro-Ile-Leu enhance the PFC number in the *in vitro* experiments when added in higher concentrations. On the other hand, Gly-Ile-Asn-Arg-Arg-Pro-Ile-Leu does not show any immunostimulative potency. However, the Ser^5-analogue of the wild type octapeptide GMNRSPIL demonstrates a strong enhancement of immunostimulative activity. The substitution of Met^2-residue in this peptide leads to the octapeptide GINRSPIL which contains two successive point mutations. Its activity is diminished as compared to GMNRSPIL and shows no dose–effect dependence.

The effects of *in vivo* immunostimulations for particular octapeptides are much less differentiated than in the *in vitro* experiments (Table 3). The wild type peptide GMNRRPIL and its Ile^2-analogue GINRRPIL show the lowest activity. There is also little difference between the GMNRSPIL and GINRSPIL action.

In the cellular immune response (DTH test), the wild type octapeptide GMNRRPIL is without any activity. Only the Ser^5-analogue (GMNRSPIL) produces strong immunosuppression (Table 4).

The influence of the wild type octapeptide GMNRRPIL and its Ser^5-analogue on the production of several cytokines is shown in Table 5. The wild type octapeptide does not influence the production of IL-1 and TNF-α, but slightly enhances the IL-6 production. On the other hand, the Ser^5-analogue does not influence IL-1 production, slightly decreases IL-6 production, but strongly increases the production of TNF-α.

With regard to IL-2 production, both examined peptides (Table 6) do not produce any distinct effect of immunomodulation.

DISCUSSION

Since the DNA-binding domain of p53 shows a distinct sequence homology to the immunostimulative thymopoietin II polypeptide from thymus, it is not surprising that a fragment of this domain, containing a thymopentin-like sequence, possesses

Table 4 The Influence of the Peptides Investigated on the Inductive Phase of DTH Reaction (Foot Pad Test) in 129/Iiw Mice Sensitized with SRBC and Treated i.p. − 3 and + 24 h after Antigen Administration with the Peptides

Peptide	Dose (μg/mouse)	Units[a]	± SE	*P* Student test
Control 0.9% NaCl	-	11.66	0.79	
GMNRRPIL	10	10.33	1.03	NS
	100	11.33	1.43	NS
AMNRRPIL	10	8.66	1.30	NS
	100	7.03	1.42	< 0.05
Control 0.9% NaCl	-	9.00	0.47	
GINRRPIL	10	7.80	0.84	NS
	100	8.30	0.71	NS
Control 0.9% Nacl	-	11.66	0.79	
GMNRSPIL	10	8.16	1.37	NS
	100	4.83	1.36	< 0.01
GINRSPIL	10	8.50	1.39	NS
	100	8.00	0.66	< 0.05

[a] 1 unit = 10^{-2} cm of the increase of foot pad thickness.

an immunostimulative activity. Similarly thymopentin, the p53 octapeptide, stimulates the humoral immune response (in PFC test), but does not influence the cellular immune response (in DTH test). The main difference between thymopentin (RKDVY) and the corresponding fragment of p53 (RRPIL) is the exchange of Asp by Pro. As we have noted above, such a substitution in TP5-like peptides very often leads to peptide immunosuppressors. This is, however, not the case for the p53 octapeptide. We

Table 5 The Influence of GMNRRPIL and GMNRSPIL Peptides, Dissolved in 0.9% NaCl, on the Production of TNF-α, IL-1 and IL-6 by P-388 D1 Cell Line[a]

Preparation	Dose (μg/ml)	TNF- α (U/ml)	IL-1 (U/ml)	IL- 6 (U/ml)
Control I Medium without LPS		7.9	8.0	8.1
Control II Medium with LPS		106.7	116.6	82.0
GMNRRPIL	1	104.8	125.3	81.9
in medium	10	113.6	126.6	92.0
with LPS	100	114.0	130.6	106.8
Control I Medium without LPS		< 4.0	32.0	16.0
Control II Medium with LPS		32.0	256.0	294.0
GMNRSPIL	1	130.0	256.0	217.0
in medium	10	141.0	256.0	264.0
with LPS	100	512.0	256.0	231.0

[a] Each result was calculated from cultures made in quadruplicate.

Table 6 The Influence of GMNRRPIL and GMNRSPIL Peptides on the Production of IL-2 by Jurkat E6.1 Cell Line[a]

Preparation	Dose (μg/ml)	IL-2 (U/ml)
Control I Medium without LPS		–
Control II Medium with LPS		26.4
GMNRRPIL in medium with LPS	1	27.8
	10	28.6
	100	26.6
Control I Medium without LPS		–
Control II Medium with LPS		28.0
GMNRSPIL in medium with LPS	1	29.0
	10	28.0
	100	30.0

[a] Peptides were dissolved in 0.9% NaCl and diluted with RPMI supplemented with 10% of FCS. Each result was calculated from cultures made in quadruplicate.

observed a similar situation for Pro^3-TP5 [22–24], which, unlike TP5 is inactive in the E-rosette and graff versus host tests, but shows some stimulative activity in the PFC test. However, as we have shown previously, the immunomodulatory activity of TP5-like peptides depends strongly on the changes in the basic N-terminal and the hydrophobic C-terminal parts of the molecule. For example, a simple exchange of the positions of two basic residues in a TP5 analogue (RKDIG vs KRDIG) transforms a peptide immunostimulant into a peptide immunosuppressor [14]. Such distinct differences regarding the immunomodulatory potency also appear in the pair of lactoferrin pentapeptides, RKPVD and RKPVT [13]. A peptide with Asp on its C-terminal is a strong immunosuppressor of both the humoral and the cellular immune response. Its analogue containing Thr in this position is active (as an immunosuppressor) in the cellular immune response only. The immunosuppressive activity was also found for the RKPKV pentapeptide, belonging to the TP5-like fragments of the transforming growth factor β1 (TFGβ1) molecule. Its analogue with the sequence RTPKV (a fragment of TGFβ3 peptide chain) demonstrates immunostimulative activity [14].

It follows that the point mutations appearing within the DNA-binding loop of p53 protein molecule change the immunodulatory activity of the respective polypeptide chain fragments. The strongest changes were in the case of $Arg^5 \rightarrow Ser^5$ substitution. The exchange of the N-terminal Gly by Ala produced a peptide with increased immunostimulative potency regarding the humoral immune response. The exchange of Met by Ile gave a product of lower activity than the wild type peptide.

The strongest changes in the immunomodulatory activity appear when the mutation takes place within the TP5-like fragment of peptide chain (Arg → Ser mutation).

However, when the additional Met → Ile mutation is present in this peptide, the product shows a distinct decrease of immunostimulation in the PFC and DTH tests. Thus, the Met → Ile mutation seems to reduce the immunomodulatory potency not only of the wild type octapeptide, but also of its Ser^5 mutant.

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