Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 787 (2003) 223–229
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Proteomic approach to apoptotic thymus maturation

Takao Kawakami[a], Takuya Nagata[b], Atsushi Muraguchi[b], Toshihide Nishimura[a,*]

[a]*Chemistry Department, Research Division, GlaxoSmithKline K.K., 43 Wadai, Tsukuba-shi, Ibaraki 300-4247, Japan*
[b]*Department of Immunology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama-shi, Toyama 930-0194, Japan*

Abstract

Apoptosis is an essential process for selection of T lymphocytes specific for foreign antigen in the process of mammalian thymus maturation. Proteomics, a comprehensive study of proteins expressed in a cell, will facilitate the systematic analysis of protein molecules related to such a complicated biological system. Protein expression profiles including information about protein signatures, localization and their quantitative changes with extracellular stimulations are extremely useful to construct intracellular pathway models resulting in the apoptotic cell death.

Keywords: Proteomics; Apoptotic thymus maturation

1. Introduction

Apoptosis is a programmed cell death depending on active participation of cellular regulation. The processing of a myriad of both external and internal impetuses leads to the apoptotic cell death, probably based on a delicate balance between them. Several mechanisms have been suggested to demonstrate the early stage of the biological processes, including Bcl-2-controlled irreversible commitment to death before so-called execution phase [1], the disruption of the inner mitochondrial transmembrane potential [2], and alteration of pH regulation mediated by mitochondria [3].

In establishment of the mammalian immune system, the apoptotic cell death is a critical process for individuals to recognize only foreign antigens. During the development of the individual, T-cell precursors in thymus undergo a complex series of differentiation steps involving the expression of two accessory molecules, called CD4 and CD8, on the cell surface, and the rearrangements of T-cell receptor (TCR) gene in the nucleus. $CD4^+8^+$ (double positive) T-cell precursors expressing low level of TCR are subjected to both positive and negative selection events [4]. Positive selection ensures the survival and differentiation of T-cells capable of recognizing a foreign antigen in the context of self-major histocompatibility complex (MHC), whereas negative selection events eliminate immature T-cell precursors expressing TCRs reacting with self-antigen by the induction of programmed cell death, apoptosis. It was demonstrated for the extracellular and cell surface events that the avidity of the interaction between their TCR and the MHC/peptide complex determines the fates of T-cell precursors for positive or negative selection [5,6]. However, the precise intracellular signaling pathway for these events is not fully understood at present.

*Corresponding author. Tel.: +81-298-645-050; fax: +81-298-648-558.

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00174-5

One of the general questions on the regulation of the apoptosis mechanism is what intracellular event(s) irreversibly commit the cell to die? It is also important to understand how signals elicited by diverse apoptosis-related proteins become integrated to initiate commitment to death.

2. Construction of a cell-free system reproducing apoptosis

The construction of a model system is the initial step to understand such a complicated biological process. Cell-free systems that essentially reproduce all of the changes seen in intact nuclei during apoptosis of T-cell precursors have been described [7–10]. It was demonstrated that engaging the CD3/TCR complex of immature mouse T-cell precursors with anti-CD3 antibody produces DNA degradation and cell death through the endogenous pathway of apoptosis [11,12]. We also found that intraperitoneal injection of anti-CD3 antibody induced apoptosis of cortical T-cell precursors.

Fig. 1 illustrates a cell-free system from the extract of T-cell precursors to reconstitute the signaling-pathway that induces apoptosis in T-cell precursors [13]. After the anti-CD3 was injected into peritonea of mice, T-cell precursors were isolated and disrupted to obtain the cell extract. The extract was incubated with nuclei isolated from liver of other normal mice. The incubation induced shrinkage of the nuclei, condensation of chromatin masses, and DNA degradation in the nuclei, observations which directly reflect the apoptotic cell death [13]. These

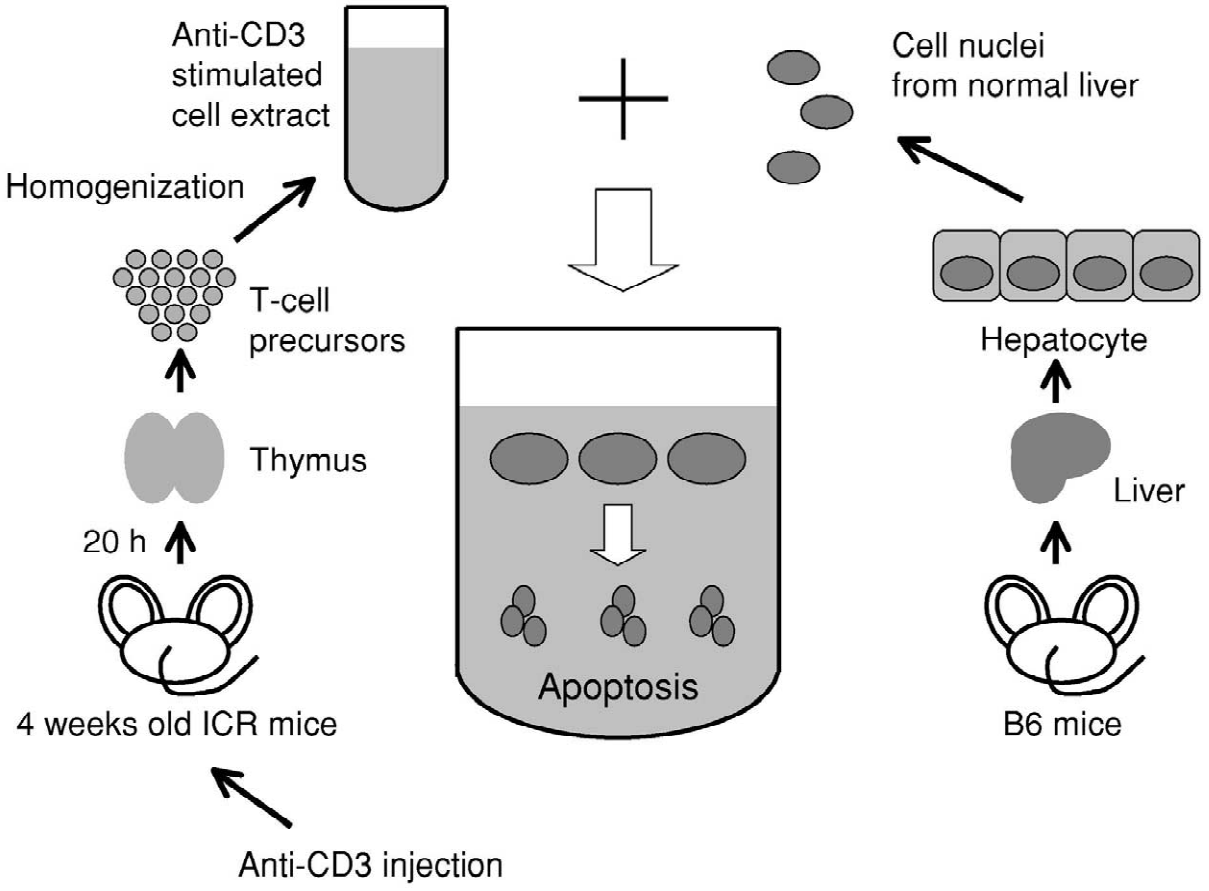

Fig. 1. Schematic representation of reconstitution of a cell-free system reproducing the changes seen in intact nuclei during apoptosis [13].

results indicate that our newly established cell-free system may reconstitute the phenomena of the death-signaling pathway of the negative selection of immature T-cell precursors in vitro.

3. Subcellular localization of apoptosis-inducing activity

The next question is in which subcellular organelles key components responsible for apoptosis-induction are localized. We separated the apoptotic cell extract by use of conventional differential centrifugation. The microsomal fraction and the high molecular mass soluble cytosolic fraction in the cell extract of anti-CD3-stimulated T-cell precursors induced DNA fragmentation in the nuclei, as found in anti-CD3-stimulated cell extract [13].

In some cases, conventional cell fractionation using a centrifuge with stepwise increasing gravity is effective to condense the protein components localized in specific organelle(s) as well as to confirm the localization of the specific biological activities. For example, when whole cell extracts are subjected to separation with two-dimensional electrophoresis (2-DE), it is difficult to identify key proteins responsible for the activity and to detect quantitative regulation of the proteins on the 2-DE gel, because such molecules exist generally in low abundance in cells. ''Cell map'' proteomics, focused on the localization study of a protein component, is one of the key areas in proteome research [14].

4. Differential display of protein expression using 2-DE gels

Without anti-CD3-stimulation, no nuclear DNA fragmentation activity was found in any fractions of the cell extract from mouse T-cell precursors. Difference of protein composition between subcellular fractions with and without stimulation by anti-CD3 should reflect the differential protein regulation by signals through TCR. Both high molecular mass cytosolic fractions from T-cell precursors with and without stimulation by anti-CD3 were compared on protein composition by 2-DE gel staining patterns (Fig. 2). After desalting with cold ethanol and acetone and dissolving with 2-DE sample buffer, both cytosolic fractions were subjected to 2-DE. 2-DE separation was carried out according to conventional methods using Immobiline DryStrip (pH 3–10, linear gradient) for the first dimensional isoelectric focusing [15]. A total of 10 spots were observed to have significant differences on their spot intensity between both silver-stained gels. These protein spots were excised from the 2-DE gels and subjected to in-gel tryptic digestion [16], followed by analysis of the resultant peptide fragments by nanoliquid chromatography (nanoLC)/nanoelectrospray (nanoESI)/ion trap mass spectrometry (MS) to identify the protein spots. Table 1 summarizes the results of identification [13].

4.1. Up-regulated proteins

The HMG2 protein was identified from four up-regulated spots (Table 1). HMG2 is one of the high mobility group proteins that associates with chromosomal DNA and induces extreme bending [17]. HMG proteins have also been observed to stimulate V(D)J cleavage by binding of RAG1 and RAG2 [18], and bind with high affinity to cisplatin-damaged DNA [19]. Toh et al. [20] reported that HMG2 enhanced the nuclease activity of DFF. We demonstrated, in apoptotic T-cell precursors, that the HMG2 was increased in the cytosolic fraction. It was also shown that the cytosolic fraction of TCR-stimulated T-cell precursors had strong nuclear DNA fragmentation-inducing activity. It is possible that the HMG2 protein is induced in cytosol by TCR-signaling and enhances the nuclease activity.

The multiple spot distribution of HMG2 may be due to the post-translational modification(s).

4.2. Down-regulated proteins

Glyceraldehyde 3-phosphate dehydrogenase is responsible for glycolysis in cytosol. Heterogeneous nuclear ribonucleoprotein A2/B1 is one of the proteins involved with pre-mRNA processing [21]. To date, the relationship between downregulation of both proteins and apoptosis induction is not clear.

Fig. 3 shows a possible pathway leading to apoptotic DNA fragmentation, expected from our studies and reports of other groups.

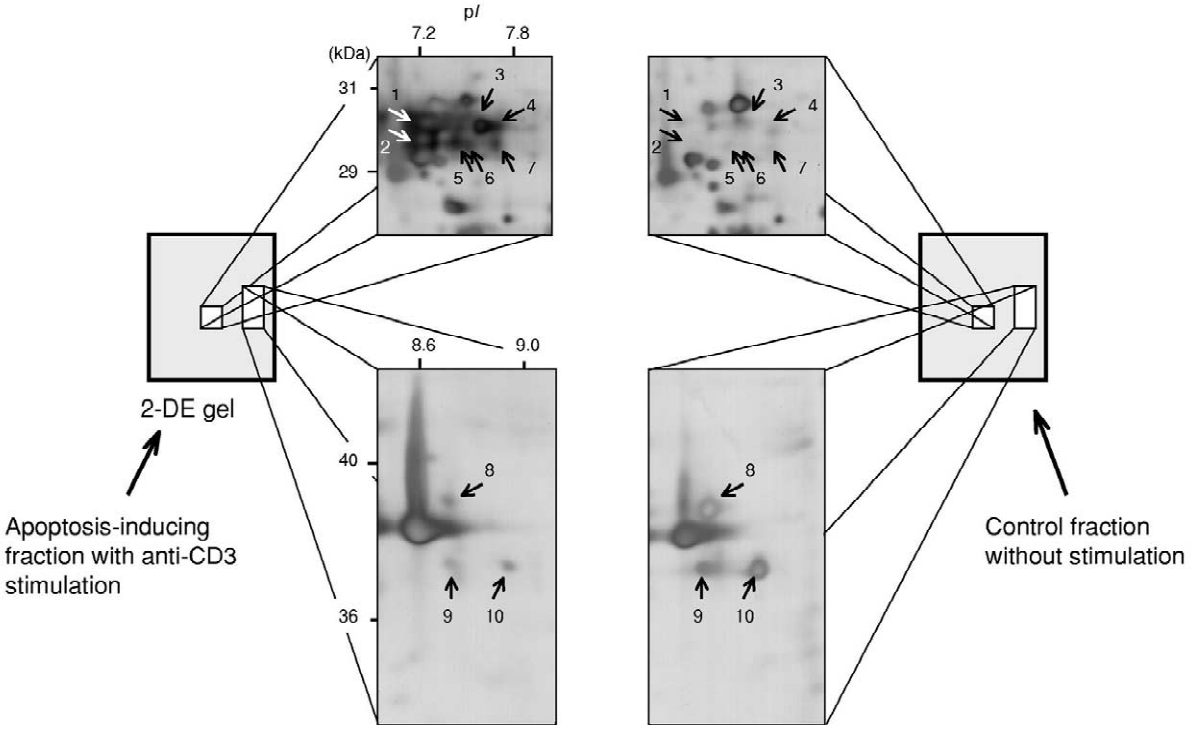

Fig. 2. Pattern comparison of 2-DE gels from both cytosolic fractions of T-cell precursors with and without anti-CD3 stimulation. This figure shows two regions of the 2-DE gels, in which protein spots with arrows were subjected to the identification process as shown in the text.

Table 1
Identified cytosolic proteins up- or down-regulated by the external stimulation of anti-CD3 antibody

Protein name	Spot no.[a]	pI/Mr (kDa)		Database accession number
		2-DE spot position	Deduced values from amino acid sequence	
Up-regulated proteins				
High mobility group protein HMG2 (four spots)	1	7.23/30.3	7.05/24.031	P30681 (SWISS-PROT)
	2	7.20/29.5		
	3	7.58/30.2		
	4	7.72/30.2		
Down-regulated proteins				
Glyceraldehyde 3-phosphate dehydrogenase (one spot)	8	8.70/39.0	8.45/35.679	P16858 (SWISS-PROT)
Heterogenous nuclear ribonucleoprotein A2/B1 (two spots)	9	8.71/36.8	8.67/35.993	AF073993 (GenBank)
	10	8.93/36.8		

[a] Spots 5, 6 and 7 were not identified.

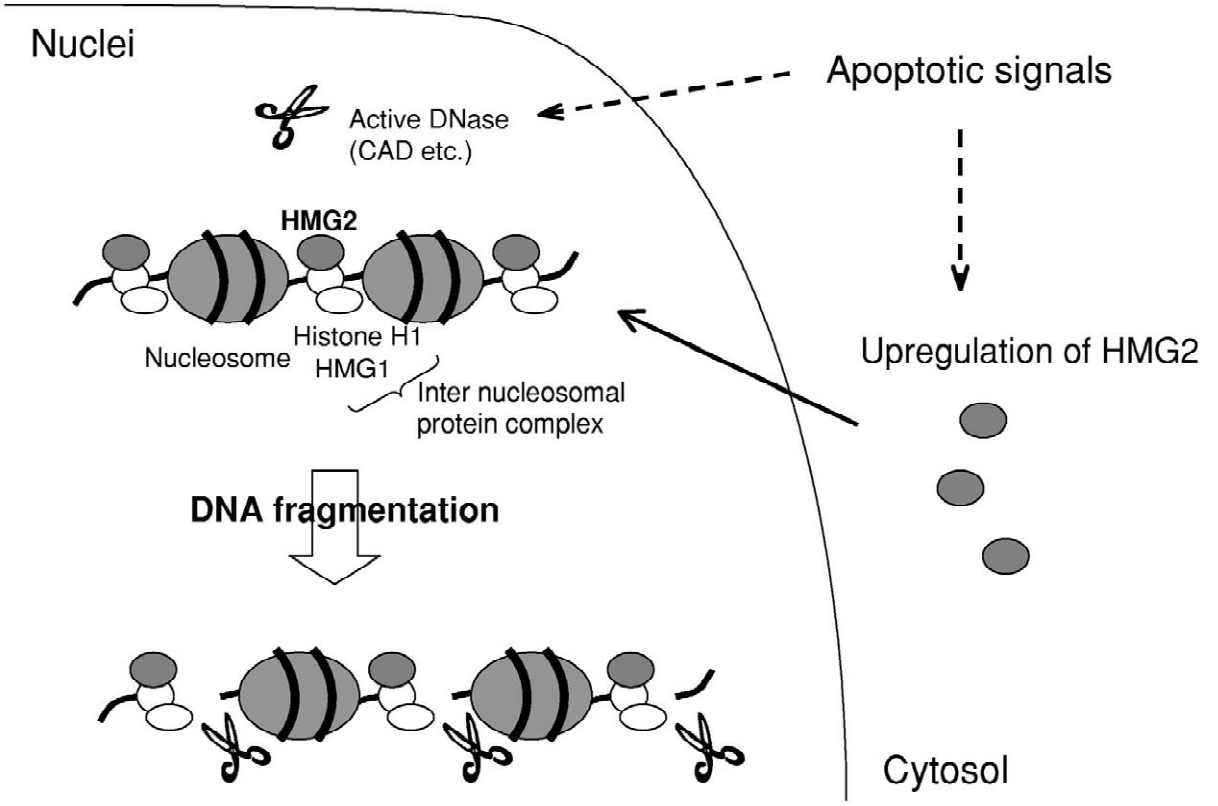

Fig. 3. Possible relationship between upregulation of HMG2 proteins in the cytosol and apoptotic DNA fragmentation in the cell nuclei.

5. Technical aspects on proteomics

At present proteomics usually uses 2-DE for protein separation, followed by analysis of the separated proteins by MS. It enables the comparison of subsets of expressed proteins among samples. 2-DE separates thousands of proteins on the polyacrylamide gel matrix according to their size and charge. Since O'Farrell [22] developed it and immobilized pH gradient (IPG) gel was introduced to the first dimension [15], 2-DE has been a useful technique with the highest resolution to survey protein expression and posttranslational modifications in cells or tissues. As shown above, proteomics is usually initiated by comparing protein compositions in an affected phase with those in a control one, in order to find proteins specifically up-regulated, down-regulated or structurally changed correlating to the affection [23].

In proteome analysis today, mass spectrometry is essential for high throughput and accurate detection/identification of proteins and it has opened a ''digital'' world in experimental biology. Many issues, including sensitivity, coverage of peptides in a protein, quantification in expression rate, and post-translational modifications such as phosphorylation, glycosylation and truncation, can be studied relating to the current proteomic mass spectrometry [24].

After the electrophoresis, proteins separated on the 2-DE gel are stained with silver nitrate or fluorescent dyes, which are currently the most sensitive detection methods. Protein spots that are different in the affected phase compared with the control phase are excised from the 2-DE gels. Excised protein spots are subjected to in situ digestion by a specific protease, usually trypsin [15]. Peptide mixtures resulting from the in-gel tryptic digestion are subjected to on-line nanoLC coupled with nanoESI/ion-trap

MS and MS/MS. The obtained peptide mass and peptide ion fragment data are searched against protein sequence databases, SWISS-PROT, PIR etc.

Proteins in a gel piece are unambiguously and simultaneously identified from peptide mass data and amino acid sequence information in the peptide ion fragments resulting from the MS/MS analysis of peptides continuously eluted from nanoLC [25].

6. Limitation of the current proteomic approach using 2-DE

However, 2-DE does not display all cellular proteins partially due to poor solubility of many hydrophobic proteins, especially membrane proteins, in the current 2-DE sample buffers. Moreover, its drawbacks include its low loading capacity, being time consuming and very labor intensive [26].

Several low abundant proteins that appear at the extremely early stage of affection may be localized and related to intrinsic functions. They seem to play key roles upstream of the signal transduction pathways regarding affection. These proteins may be hidden under house-keeping proteins of huge abundance or simply not be detectable on the 2-DE gel. The detection limit is around 5 ng by fluorescent dye or silver nitrate, currently the most sensitive staining methods. One can easily imagine that those proteins are located in ''white'' areas on the 2-DE gel display or overlap with abundant protein spots.

On the protein solubility, protein profiling can be improved by the combination of one-dimensional sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and the above nanoLC/nanoESI/MS/MS [27]. Prior to electrophoresis, most proteins extracted from tissue or cell samples are dissolved and denatured in the sample buffer by the strong anionic detergent, SDS. After the electrophoresis gel is cut into slices, and peptide mixtures resulting from in-gel tryptic digestion of each slice are subjected to on-line nanoLC/MS/MS to identify authentic proteins. SDS–PAGE has been widely used for protein separation and molecular mass estimation [28], as its easy handling allows most proteins, including hydrophobic ones, to be successfully applied. These advantages mean that the combination of SDS–PAGE fractionation and nanoLC/MS/MS analysis provides a useful alternative to conventional 2-DE-based techniques. The strategy improves the identification of hydrophobic membrane proteins impossible to analyze by use of 2-DE.

7. Other approaches to the apoptotic signal transduction pathway

Many investigators reported on the signaling pathway or molecules responsible for positive selection or negative selection of T-cell precursors. ZAP-70, one of the protein tyrosine kinases that is involved in the activation of T lymphocytes, was reported to be essential for both positive and negative selection [29]. It was also demonstrated that the Ras/MAP kinase pathway and the calcineurin pathway is necessary for positive selection, but not for negative selection [30–33]. An obvious interpretation emerging from these studies is that events proximal to the TCR may be needed for both positive and negative selection, but that other events further downstream of the TCR may determine the outcome of receptor engagement.

8. Conclusions and future perspectives

We have established, for the first time as far as we know, a novel cell-free system in which extract of T-cell precursors that received signals through TCR molecules induces apoptotic morphological changes and DNA fragmentation in the naked nuclei.

Several proteins regulated differentially in the cytosol of T-cell precursors by a signal from TCR were identified by introduction of proteomic techniques. Such protein molecules responsible for signal transduction through cell membranes or between cells are generally few in number. Conventionally, the active fraction can be further subjected to fractionation with several steps of column-separation in order to purify the molecule(s) responsible for the activity. However, these purification procedures may cause considerable loss of the target protein(s). In the proteomics approach, it may be possible to perform all-inclusive screening of protein differential regulation and structural changes such as phosphorylation and glycosylation without loss of the proteins dissolved in the sample buffer for 2-DE separation. Moreover, using nanoLC/nanoESI/MS/MS for micro-

characterization leads to high throughput protein screening and identification. Using these techniques, several proteins were observed to be up- or down-regulated by the signals from the TCR, and an apoptosis-related HMG2 protein was identified. Although current proteomic techniques using 2-DE gels have several limitations to be overcome as shown above, the present approach is potentially useful to understand the protein regulation in the intracellular signaling pathway leading to apoptosis as well as identification of key protein(s) for apoptosis-induction.

Several proteome profiling methods, the gel-based one as shown above or a chromatography-based one [34], can be alternatives to 2-DE. Application of these to the present study may be useful for further characterization and isolation of molecules responsible for the death-signaling pathway in the self-destructive autoreactive T lymphocytes.

9. Nomenclature

2-DE	two-dimensional electrophoresis
MHC	major histocompatibility complex
MS	mass spectrometry
NanoESI	nanoelectrospray ionization
NanoLC	nanoliquid chromatography
TCR	T-cell receptor

References

[1] G.J. Griffiths, L. Dubrez, C.P. Morgan, N.A. Jones, J. Whitehouse, B.M. Corfe, C. Dive, J.A. Hickman, J. Cell Biol. 144 (1999) 903.
[2] L. Schild, R.M. Matthias, A. Stanarius, G. Wolf, W. Augustin, W. Halangk, Mol. Cell. Biochem. 195 (1999) 191.
[3] S. Matsuyama, J. Liopis, Q.L. Deveraux, R.Y. Tsien, J.C. Reed, Nat. Cell. Biol. 2 (2000) 318.
[4] H. von Boehmer, P. Kisielow, Science 248 (1990) 1369.
[5] E. Sebzda, V.A. Wallace, J. Mayer, R.S. Yeung, T.W. Mak, P.S. Ohashi, Science 263 (1994) 1615.
[6] P.G. Ashton-Rickardt, A. Bandeira, J.R. Delaney, L. Van Kaer, H.P. Pircher, R.M. Zinkernagel, S. Tonegawa, Cell 76 (1994) 651.
[7] Y.A. Lazebnik, S. Cole, C.A. Cooke, W.G. Nelson, W.C. Earnshaw, J. Cell Biol. 123 (1993) 7.
[8] D.D. Newmeyer, D.M. Farschon, J.C. Reed, Cell 79 (1994) 353.
[9] S.J. Martin, D.D. Newmeyer, S. Mathias, D.M. Farschon, H.G. Wang, J.C. Reed, R.N. Kolesnick, D.R. Green, EMBO J. 14 (1995) 5191.
[10] M. Enari, A. Hase, S. Nagata, EMBO J. 14 (1995) 5201.
[11] C.A. Smith, G.T. Williams, R. Kingston, E.J. Jenkinson, J.J. Owen, Nature 337 (1989) 181.
[12] Y.F. Shi, R.P. Bissonnette, N. Parfrey, M. Szalay, R.T. Kubo, D.R. Green, J. Immunol. 146 (1991) 3340.
[13] T. Kawakami, T. Nagata, A. Muraguchi, T. Nishimura, Electrophoresis 21 (2000) 1846.
[14] A.W. Bell, M.A. Ward, W.P. Blackstock, H.N.M. Freeman, J.S. Choudhary, A.P. Lewis, D. Chotai, A. Fazel, J.N. Gushue, J. Paiement, S. Palcy, E. Chevet, M. Lafreniere-Roula, R. Solari, D.Y. Thomas, A. Rowley, J.J.M. Bergeron, J. Biol. Chem. 276 (2001) 5152.
[15] A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037.
[16] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Anal. Chem. 68 (1996) 850.
[17] T.T. Paull, M.J. Haykinson, R.C. Johonson, Genes Dev. 7 (1993) 1521.
[18] D.C. Van Gent, K. Hiom, T.T. Paull, M. Gellert, EMBO J. 16 (1997) 2665.
[19] J.E. Cryer, S.W. Johonson, B.N. Engelsberg, P.C. Billings, Cancer Chemother. Pharmacol. 38 (1996) 163.
[20] S.Y. Toh, X. Wang, P. Li, Biochem. Biophys. Res. Commun. 250 (1998) 598.
[21] http://www.ncbi.nlm.gov/entrez
[22] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[23] T. Kawakami, T. Nishimura, J. Clin. Exp. Med. 196 (2001) 428.
[24] T. Nishimura, T. Kawakami, Y. Maruyama, Y. Fujita, Genes Med. 4 (2000) 617.
[25] T. Kawakami, F. Usui, T. Nishimura, Jpn. J. Electrophoresis 44 (2000) 185.
[26] T. Kawakami, H. Anyoji, T. Nishimura, J. Mass Spectrom. Soc. Jpn. 50 (2002) 135.
[27] T. Kawakami, H. Anyoji, Y. Kasahara, N. Ogasawara, K.D. Nugent, F. Usui, Y. Bando, T. Nishimura, in: Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, 2001, WPI178.
[28] U.K. Laemmli, Nature 277 (1970) 680.
[29] I. Negishi, N. Motoyama, K. Nakayama, K. Nakayama, S. Senju, S. Hatakeyama, Q. Zhang, A.C. Chan, D.Y. Loh, Nature 376 (1995) 435.
[30] J. Alberola-Ila, K.A. Forbush, R. Seger, E.G. Krebs, R.M. Perlmutter, Nature 373 (1995) 620.
[31] J. Alberola-Ila, K.A. Hogquist, K.A. Swan, M.J. Bevan, R.M. Perlmutter, J. Exp. Med. 184 (1996) 9.
[32] K.A. Swan, J. Alberola-Ila, J.A. Gross, M.W. Appleby, K.A. Forbush, J.F. Thomas, R.M. Perlmutter, EMBO J. 14 (1995) 276.
[33] C.R. Wang, K. Hashimoto, S. Kubo, T. Yokochi, M. Kubo, M. Suzuki, K. Suzuki, T. Tada, T. Nakayama, J. Exp. Med. 181 (1995) 927.
[34] T. Kawakami, H. Anyoji, T. Nishimura, Proteome Digest, Fall issue (2002) 14.