Serum Ubiquitin Concentration in Patients with Acute Myocardial Infarction

AKUT MİYOKARD ENFARKTÜSLÜ HASTALARDA SERUM UBIQUITIN KONSANTRASYONU

Ibrahim PİRİM*, Kenan GÜMÜŞTEKİN**, Yaşar Nuri ŞAHİN*

* Dept. of Biochemistry, Medical School of Atatürk University,
** Dept. of Physiology, Medical School of Atatürk University, Erzurum, TURKEY

Summary

The aim of this study was to design a simple method for detecting the ubiquitin protein in serum samples from AMI patients and evaluate its diagnostic and prognostic value. For this aim, sandwich type ELISA assay was used.

Serum concentration of ubiquitin was found to be increased in acute myocardial infarction (AMI) patients following severe chest pain at 6th, IOth, 17th and 30th hours compared to healthy subjects. Therefore, detection of serum ubiquitin level may have diagnostic and prognostic value in AMI, together with enzymes that are specific for heart muscle.

Key Words: Ubiquitin, Acute Myocardial Infarction, ELISA

T Klin J Med Res 2000, 18:1-5

Özet

Çalışmanın amacı akut miyokard enfarktüstü hastaların serumlarında ubiquitin proteinini tesbit etmek için basit bir yöntem geliştirmekti. Sandwich-ELISA yöntemi, ubiquitine karşı üretilmiş monoklonal ve poliklonal antikorları kullanarak ubiquitini serumda ölçmek için uyarlandı.

Çalışmamızın sonucunda kontrollerle kıyaslandığında göğüs ağrısını takip eden 6, 10, ¡7 ve 30. saatlerde ubiquitin seviyesinin yüksek olduğu bulundu. Sonuç olarak serum ubiquitin seviyesinin diğer kalp kasına spesifik enzimlerle beraber kullanıldığında diagnostik ve prognostik bir değerinin olabileceği düşünüldü.

Anahtar Kelimeler: Ubiquitin, Akut Miyokard Enfarktüsü, ELISA

T Klin Araştırma 2000, 18:1-5

Ubiquitin, a 76 amino acid protein, is present in the cytoplasm and nucleus of eucaryotic cells and can be covalently conjugated to cellular proteins by the enzymes of the ubiquitin conjugating system (1,2). In this process, several ubiquitin monomers are usually ligated sequentially to another ubiquitin moiety already linked to the protein, forming multiubiquitin chains (3). The multi-ubiquitin chain acts as a signal to induce degradation of the target proteins by 26S proteasome (2,3). The ubiquitin mediated proteolysis, a major pathway for selective protein degradation, plays a variety of regulatory roles in cellular processes, including stress response (4,5),cell cycle (6,7), gene expression (8) and apoptosis (9). Ubiquitin may additionally be involved in the pathogenesis of various diseases. Intracellular accumulation of ubiquitin has been detected in patients with neurodegenerative diseases (10), muscular diseases (11), brain ischemia (12), cancer (13), rheumatoid arthritis and hemodialysis (14). Extracellular ubiquitin has been suggested to be involved in amyloid formation (15) and growth of hematopoietic cells (16). Several studies have revealed that ubiquitin concentration in body fluids are increased in patients with various diseases: serum ubiquitin in parasitic and allergic disease (17), plasma ubiquitin in chronic renal failure, especially in cases undergoing hemodialysis treatment (14,17), and cerebrospinal fluid ubiquitin in Creutzfeld-Jacop disease (18) and Alzheimer's disease (19,20). However, origin and metabolism of ubiquitin in the body fluid have not been clarified yet.

Received: Dec. 15, 1998

Correspondence: Ibrahim PİRİM
Dept. of Biochemistry,
Medical School of Atatürk University,
25240 Erzurum, TURKEY

The aim of this study was to design a simple method for detecting the ubiquitin protein in serum samples from AMI patients and evaluate its diagnostic and prognostic value. For this aim, sandwich type ELISA assay was used.

Materials and Methods

Serum Collection

Blood was obtained from patients (11 male 42 17; 8 female 54 11) who were admitted to Cardiology Department of University hospital and General State hospital. Serum collection was performed according to onset of severe chest pain. Since the patients' arrival to the hospital varied with regard to onset of severe chest pain, time of blood collection from patients was adjusted. Protocol for blood collection from patients is given in Table 1. We also collected blood from 39 healthy volunteers for assessment of ubiquitin quantitation.

Commercially available anti-ubiquitin antibody, purified ubiquitin protein and peroxidase conjugated rabbit immunoglobulins to mouse immunoglobulins (PRAM) were purchased from sigma (U5379, U6253 and A9044 respectively). Monoclonal anti-ubiquitin antibody was kindly provided by R. Layfield (Noitingham Univ., QMC, UK).

Assay Procedure

We have designed sandwich-type ELISA assay for ubiquitin 96-well Maxisorp plates, (Cat. No 469949; Nunc Roskilde, Denmark) were passively coated at 37°C for 24 h with polyclonal anti-ubiquitin antibody (200ml/well). After washing with washing buffer (distilled water containing 0,5 ml/1 Tween 20), 100ml of purified ubiquitin protein in different concentrations (concentration range 1 ng/ml-30 ng/ml), and 100ml serum sample of control group and patients with AMI (neat) were added to the wells. Later 100 ml monoclonal anti-ubiquitin antibody (1:750 dilution) was added to each well. We then added to each well 100 ml of peroxidase labeled anti mouse immunoglobulin antibody (1:500 dilution). After 4 h incubation and washing 10 times with 300 ml of washing buffer, we added to each well 200 ml of substrate solution (0.1% w/w ABTS (2,2 azino-bis, 3-ethylbenzthiazoline-6-sulphonic acid), 0.003% v/v H202 (100 vol) in citrate/phosphate buffer pH 4). After incubation for 15 min, the absorbance of the plates was read at 405 nm with a spectrophotometer. The color change was proportional to the amount of antigen in the test solution.

A sequential multiple automated analyzer (Hitachi 717) determined the following analytes in the serum specimens from AMI patients depending on the times (see Table 1): total creatine kinase (CK), creatine kinase isoenzyme (CK-MB) and total lactate dehidrogenase (LDH).

Results

Passively coated plates with polyclonal anti-ubiquitin antibody were used for patients with AMI and for healthy controls to establish reference range of serum ubiquitin concentration. In order to draw

Table 1. Mean SD values of serum ubiquitin CK, CK-MB and LDH in patients with Acute Myocardial Infraction at different time points after the onset of severe chest pain (n:19).

TIME	UBIQUITIN (9.4 3 ng/ml)*[3]	CK (0-190 U/L)*	CK-MB (0-25 U/L)*	LDH (225-450U/L)*
3rd h	9.8 2.41	96 56	16 13	229 40
4th h	10.53 1.93	156 46	23 15	271 57
6th h	12.94 2.09 #	274 123	67 38	345 38
10th h	19.0 3.76 #	722 237	167 86	572 221
17th h	26.7 4.66 #	1176 305	413 226	879 317
30th h	21.8 3.92 #	1489 370	598 176	1397 630

* Reference range

[3] Reference value for ubiquitin was obtained from 39 healthy volunteer subjects

Compared with reference value ($p<0.05$)

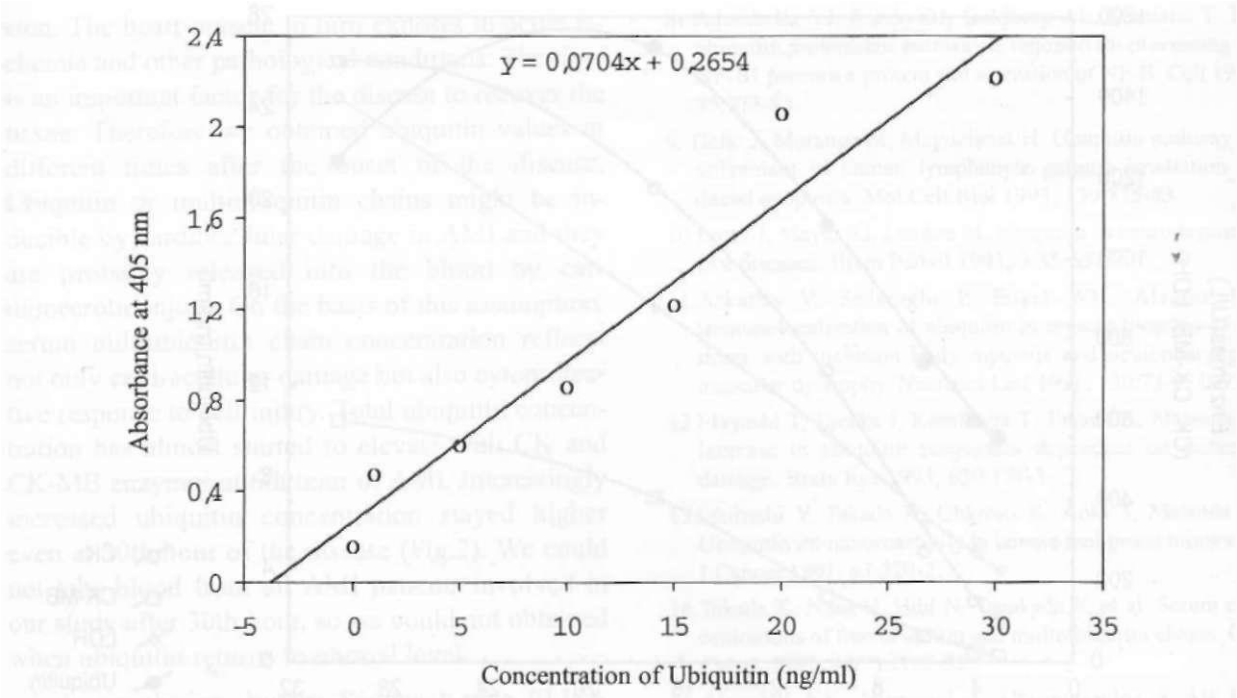

Figure 1. Standard dilution curve of ubiquitin obtained by using sandwich type ELISA.

standard curve, ubiquitin (U6253, Sigma) in different concentrations (30ng/ml, 20ng/ml, 15ng/ml, lOng/ml, 5ng/ml and 1 ng/ml) and monoclonal anti-ubiquitin antibody (1:750) was used. Absorbance versus amount of antigen graph was drawn (see Figure 1).

In order to establish reference range of ubiquitin by using sandwich-type ELISA assay, we analyzed the serum concentration of ubiquitin in 39 healthy volunteers (mean age 31±18). There was no significant correlation between age and ubiquitin concentration (data not shown). Reference range of ubiquitin concentration for our assay was compared to standard ubiquitin dilution curve. Concentration of ubiquitin and cardiac enzymes in serums taken according to the time intervals of 19 AMI patients showed significant increase. Specially while CK and CK-MB has started to increase at the 3th and 4th hour of AMI, ubiquitin concentration appeared to be in the normal range of established ubiquitin reference values. LDH was also in the normal level. However, ubiquitin has started to elevate at 6th hour and stayed at higher concentrations at 10th ,17th and 30th hour of AMI. Although ubiquitin concentration has seemed to be declining at 30th hour of AMI, it was, however significantly higher than control value (see Table 1 and Figure 2).

The normal ubiquitin values obtained from 39 healthy control were statistically compared with 19 AMI patients' 6th, 10th , 17th and 30th hour ubiquitine values. Statistical analysis were performed by student's t-test. P values were less than 0.05 and considered to be statistically significant.

Discussion

At least two distinct types of antibody against ubiquitin are sold commercially. One is available from DAKO, Denmark has been produced using SDS-denatured ubiquitin linked to chicken gamma-globulins with glutaraldehyde according to Haas and Bright (21). The other which is available from Sigma has been raised against ubiquitin conjugated to keyhole limpet haemocyanin and readily react with free ubiquitin as well as with conjugates. In raising antibodies against ubiquitin, conjugation is important because it is not very antigenic in its free state due to the compact structure and highly conserved sequence. When conjugated, it is thought that it undergoes a conformational change and the structure opens up exposing particular epitopes

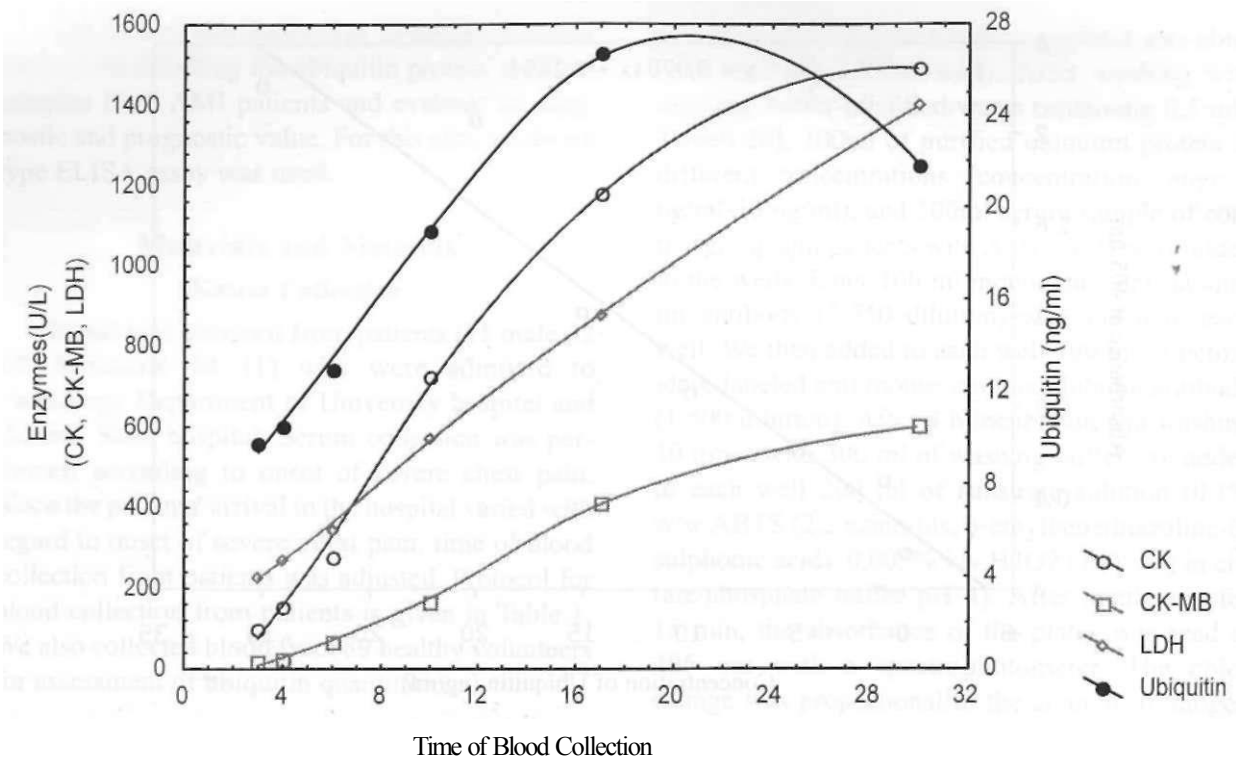

Figure 2. Serum concentrations of total ubiquitin and cardiac enzymes (CK, CK-MB, LDH) in patients with AMI. Graph values are from Table 1.

(22). This proposal is supported by the existence of antibodies that preferentially bind to free or to conjugated forms of the protein (21). Here purified ubiquitin protein has been used to identify ubiquitin concentration for standard curve graph. The antibody recognizes either free or conjugated ubiquitin. Structure of ubiquitin within the control and AMI samples could not be established. In this sandwich-type ELISA assay for ubiquitin, antibody is labeled with classical peroxidase (DAKO, Denmark, PRAM). The peroxidase labeled Fab' fragment of antibody is directed against monoclonal antibody which is against ubiquitin and the immobilized polyclonal antibody coated onto the surface of the wells of 96-well plates directed against ubiquitin protein. The assay is quite sensitive, detecting lng ubiquitin Per well. The interassay reproducibility tests carried out with 39 control samples were in range of values which we obtained.

This study of 39 control subjects of both sexes between ages 12 and 57 has allowed as to determine the reference value of ubiquitin. Obtained ubiquitin serum values was total ubiquitin, including both free and conjugated form of it. However in recent work, free and conjugated ubiquitin reference values within the normal serum has been determined (14). In the ubiquitin mediated proteolytic pathway, free ubiquitin and multiubiquitin chains have distinct cellular roles: the former is a pool for future conjugation to cellular proteins and the latter is an active form that induces their degradation (3). Thus the measurement of both ubiquitin forms are informative for deducing the cellular state of the ubiquitin system. We could have, however, determined only total ubiquitin by sandwich type ELISA. The method was quick, sensitive and specific. Takada et al. measured and quantified both ubiquitin forms in the cells (14). Okada et al. reported increased plasma ubiquitin in hemodialysis patients (23). The other works have also reported that serum concentration of multiubiquitin chains increases in acute viral hepatitic patients (14),in acute stress i.e. heat shock (4) and in ischemia (12). In our study, we aimed to follow ubiquitin levels in AMI according to time intervals of the disease onset. AMI occurs in case of coronary artery occlu-

sion. The heart muscle in turn exposes to acute ischemia and other pathological conditions. The time is an important factor for the disease to recover the tissue. Therefore we obtained ubiquitin values at different times after the onset of the disease. Ubiquitin or multiubiquitin chains might be inducible by cardiocellular damage in AMI and they are probably released into the blood by cardionecrotic injury. On the basis of this assumption, serum miltiubiquitin chain concentration reflects not only cardiocellular damage but also cytoprotective response to cell injury. Total ubiquitin concentration has almost started to elevate with CK and CK-MB enzymes at 6th hour of AMI. Interestingly increased ubiquitin concentration stayed higher even at 30th hour of the disease (Fig.2). We could not take blood from all AMI patients involved in our study after 30th hour, so we could not obtained when ubiquitin returns to normal level.

In conclusion, by the Sandwich-type ELISA method described in this study, it is possible to measure the serum ubiquitin level, and it may have a diagnostic and prognostic value with the other cardiac enzymes in AMI patients.

REFERENCES

1. Hershko A, Heller H, Ellias S, Ciechanover A. Components of ubiquitin protein ligase system. Resolution affinity purification and role in protein breakdown. J Biol Chem 1993; 258:8206-14.
2. Hershko A, Ciechanover A. Ubiquitin system for protein degradation. Annu Rev Biochem 1992; 61:761-807, 1993.
3. Chau V., Tobias JW., Bochmair A., Marriott D., Clonda DK. A multiubiquitin chain is confined to specific lysine in a targeted short lived protein. Science. 1989; 243:1576-83, 1993.
4. Parag HA., Raboy B., Kulka RG. Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system. EMBO J 1987; 6:55-61.
5. Finley D, Ozkaynak E, Varshavsky A. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell 1987; 48:1035-46.
6. Funabiki H, Yamano H, Kumada K, Nago K, Hunt T, Yanogida M. Cut2 proteolysis required for sister chromatid separation in fission yeast. Nature 1996; 351:438-41.
7. Glatzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature 1991; 349:132-8.
8. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin proteosome pathway is required for processing the NF-B1 precursor protein and activation of NF-B. Cell 1994; 75:773-85.
9. Delic J, Morange M, Magdclcnat H. Ubiquitin pathway involvement in human lymphocyte gamma irradiation induced apoptosis. Mol Cell Biol 1993; 13:4875-83.
10. Lowe J, Mayer RJ, Landon M. Ubiquitin in neurodegenerative diseases. Brain Pathol 1993; 3:55-65.
11. Askanas V, Sedaroglu P, Engel WK, Alvarez RE. Immunolocalization of ubiquitin in muscle biopsies of patients with inclusion body myositis and oculopharyngeal muscular dystrophy. Neurosci Lett 1991; 130:73-6, 1993.
12. Flayashi T, Tanaka J, Kamikuba T, Takada K, Matsuda M. Increase in ubiquitin conjugates dependent on ischemic damage. Brain Res 1993; 620:171-3.
13. Ishibashi Y, Takada K, Ohkowa K, Aoki T, Matsuda M. Ubiquitin immunoreactivity in human malignant tumors. Br J Cancer 1991;63:320-2.
14. Takada K, Nasu H, Hibi N, Tasukada Y, et al. Serum concentrations of free ubiquitin and multiubiquitin chains. Clin Chem 1997; 43(7): 1188-95.
15. Alizadeh KK, Normand J, Chronopoulos S,.Ali KZ. Amyloid enhancing factor activity is associated with ubiquitin. Virchows Arch. A Pathol Anat Histopathol 1992; 420:139-48.
16. Daino H, Shibayama H, Machii T, Kitani T. Extracellular ubiquitin regulates the growth of human hematopoietic cells. Biochem Biophys Res Commun 1996; 223:226-8.
17. Asseman C, Pancre V, Delanoye A, Capron A, Auriault C. A radioimmunoassay for the quantification of human ubiquitin in biological fluids: application to parasitic and allergic diseases. J. Immunol Methods 1994; 173:93-101.
18. Monaka H, Kato T, Kurita K, Katagiri T, Shikamo Y, Kujiral K. Marked increase in cerebrospinal fluid ubiquitin in Creutzfeldt Jakop disease. Neurosci Lett 1992; 139:47-9.
19. Kudo T, Iclbai K, Ravid R, Swaab DF. Alzheimer's disease, correlation of CSF and brain ubiquitin levels. Brain Res 1994; 639:1-7.
20. Wang GP, Igbal K, Bucht G, Winblad B, Wisniewski HM. Alzheimer disease. Paired Helical Filaments immunoreactivity in cerebro spinal fluid. Acta Neuropathol 1991; 82:6-12.
21. Haas A and Bright P. The immunochmical detection and quantitation of intracellular ubiquitin protein conjugates. J Biolog Chem 1985; 260(23): 12464-70.
22. Wilkinson KD and Mayer A. Alcohol induced conformational changes of ubiquitin. Arch. Biochem Biophys 1986; 250:378-83.
23. Okada M, Miryazaki S, Hirasawa Y. Increase in plasma concentration of ubiquitin in dialysis patients. Clin Chim Acta 1993; 220:135-44.