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키토산 유도체인 Sulfated N-acetyl Chitosan의 종양전이 억제효과

†류 병 호 · 김 동 석 · *필립 그린스판
†경성대학교 공과대학 식품공학과 · 조오지아 주립대학교 약학대학 약학과

Inhibitory Effects of Tumor Metastasis by Chitosan Derivative, of Sulfated N-acetyl Chitosan

Beung-Ho Ryu†, Phillip Greenspan*
†Department of Food Technology and Microbiology · Kyungsung University, Pusan 608-736, Korea
*College of Pharmacy, The University of Georgia, U. S. A.

ABSTRACT

Chitosan derivative, of a sulfated N-acetyl chitosan was synthesized, and the inhibitory effects of this compound on the experimental and spontaneous lung metastatic B16/BL6 melanoma bearing mice were investigated. Position of substitution with sulfate in water-soluble sulfated derivatives of chitosan were analysed by ^{13}C-nmr. The structure of N-acetyl chitosan 3,6 O-disulfate were confirmed. The tumor growth inhibition of B16/BL6 melanoma cells has been shown at the highest level of 77.6% when sulfated N-acetyl chitosan were administered at the dose of 100mg/kg. In the lung metastasis, the sulfated N-acetyl chitosan was administered to C57BL/6B mice bearing B16/BL6 melanoma cells by I.V. injection and the number of metastasis foci of melanoma were decreased by the dose dependent manner ranging from 20 to 100mg/kg. In the spontaneous metastasis, I.V. administrations of sulfated N-acetyl chitosan after tumor inoculation resulted in marked reduction of metastatic colonies. A sulfated N-acetyl chitosan was able to partially inhibit the tumor cell adhesion by migration to laminin. These results suggested that chitosan derivatives, a sulfated N-acetyl chitoasn was able to inhibit to the experimental and spontaneous metastasis models as well as cell adhesion ability.

INTRODUCTION

Tumor cells metastasis is a complicated phenomenon that involves one of the major causes of mortality in cancer. The sequential steps of tumor cells metastasis interact with various hosts cells such as platelets, lymphocytes, endothelials and with extracellular matrix components such as fibronectin, laminin, collagen and sulfated glucosaminoglycan(1-5). In these processes, the adhesion of tumor cells to these extracellular matrix component is an important step of the metastasis pathway, in which some specific interaction between tumor cells adhesion receptor and com-

† Corresponding Author

ponents of extracellular matrix is involved(6-9). This adhesive interaction may lead to the enhancement of survival, arrest or invasiveness of tumor cells and is an important event in the metastasis cascade(8-13). Some scientific approach has been carried out to regulate the mechanism involved in cells adhesive interaction during the matastatic process for anti-adhesion therapy of cancer(14, 15). Some sulfated glycosaminoglycans derived from extracellular matrix and basement membrane have been found to modulate the mechanism involved in metastasis of tumor cells.

Interestingly, sulfated polysaccharide as a polyanionic compounds may exert antimetastasis activity by several mechanisms. Dextran sulfate (16, 17), chondroitin sulfate(18), and heparan (19) as sulfated polysaccharides inhibited the metastasis of tumor cells and the inhibition might be partially due to its effect on changing the ionic properties of tumor cells surface. Also sulfated polysaccharides was able to inhibit the blood borne pulmonary metastasis and tumor emboli by platelet aggregation at the stage of tumor lodgement(21).

On the other hand, chitin and chitosan derivatives are widely distributed in nature, which are crustacean, insects, mushroom and in the cell wall of bacteria(20), and have been reported to have some beneficial medicinal(21) and pharmacological application(22).

Chitin and chitosan derivatives have been reported to show the biological function of controlled releases or bioavailability of drugs(23), and wound-healings(24), antibacterial(25), antivirus (26), and antitumors(27). Especially, the sulfate of chitin derivatives showed low levels of anticoagulants and antiplatelet aggregation activities and was found to be effective in the prevention of metastasis in mouse metastatic cancer model(28,29). Here we know that there is the effect of sulfated N-acetylchitosan(SNAC).

We investigated to the effect of sulfated N-acetylchitoasn against metastasis of tumor cells. It's the deacetylation derivative of chitin which is a unbranched polymer with structure of 2-N-acetyl 2-deoxy β(1-4) D-glucan. We found that sulfated N-acetyl chitosan has antimetastasis activity of tumor cells and cell adhesion with no significant toxic effects on cultured cells.

Materials and Method

Chitosan (Flonac-N, commercial chitosan of crab shell, d.s. for HAC.[α], -5℃) was obtained from Kyowa Yushi Co. Flonac -N was treated with aqueous 40% NaOH containing $NaNH_4$(0.5/500ml) at 110℃ for 5 hrs to afford purified product (d.s. 0.05 for HAc) which had[α], at -10℃ 2% aqueous acetic acid.

N-acetylation of chitosan was performed according to the method of Hirano and Kinugawa (30).

Sulfation of N-acetylated chitosan

Sulfation of chitosan was carried out according to the method of Schweiger(31). N-acetyl derivative of chitosan(0.5gr) was dissolved in methane sulfonic acid(5ml) in an ice bath or swollen in N, N-dimethylformamide(5mL) at room temperature, and sulfated with SO_3-DMF complex (5-7 mole equivalent amounts to one hydroxyl group) by stirring at room temperature overnight. The reaction mixture was poured into ice cooled water, and the solution was adjusted to pH 9 with 2.5 N-NaOH. Three volumes of 95% ethanol were then added, the precipitate produced was collceted by means of centrifugation(2,000×g, at 20 min) and dissolved in a small volume of water. The solution was dialyzed against distilled water, overnight, and lyophilized to afford the sulfated products of N-acetyl chitosan.

Spectrum of IR and ^{13}C-n. m. r.

Infra-red spectra(KBr) were recorded with Hitachi 215 grating spcetrometer, and 13C-n.m.r. spectra with a Jeol=FX 200 FH-NMR

Animal and tumor cells

Specific pathogen-free female C57BL/6Bmice at three weeks age were used. Animals were fed

a commercial pellet diet with water *ad libitum*.

Cell culture

A highly metastatic cells, B16/BL6 mouse melanoma was generously provided by Anderson Hospital Cancer Center(Houston, U. S. A.). The cells were maintained as monolayer culture in Eagle's minimal essential medium(EMEM) (Sigma Co)supplemented with 10% Fetal bovin serum(FBS), vitamin solution, sodium pyruvate, nonessential amino acid and L-glutamate.

Tumor growth assay

Tumon nodules of B16/BL6 melanoma were collected for 10 days after i. v. injection of 1×10^5 cells into C57BL/6B mice, and 0.1mL of 1:4 tumor homogenate in 0.9% Nacl solution was transplanted into C57B1/6B mice on the first day (32). The tumor was weighed on the day 13.

Microassay for cell adhesion

The cells attachment assay was performed according to the method of Murata, et. al. (35). B16/BL6 melanoma cells in an exponential growth phase were incubated for 24hrs in EMEM containing FBS supplemented with 0.4uCi/ml [^{125}I] iododeoxyuridine([^{125}I]-IUdR) (specific activity, 200mCi/mmole, New England Nuclear, Boston. U. S. A.). The cells were washed twice in warm saline phosphate buffer(PBS) to remove unbound radiolabel, harvested by adding 0.02%-EDTA for 1min at 37℃, and resuspended in cold serum free EMEM to form a single cell suspension. [^{125}I] IUdR-labeled tumor cells(1.0×10^4) in a volume of 0.05ml/well were added to microculture cells precoated with laminin. The cultures were incubated at 37℃ for 30mins and then the wells were washed four times with PBS to remove unattachment cells. The remaining substrate-bound tumor cells were lysed with 0.1N-NaOH. The lysate was absorded on cotton swab and monitored for radioactivity by gamma counting. The binding capacity(No. of cells bound/substrate) was expressed as follows:

$$\text{Binding capacity}=\frac{\text{cpm of targets bound to substrate}}{\text{cpm of total tumor cells added}}\times\frac{}{\text{total number of tumor cells added}}$$

Assay for pulmonary matastasis

Experimental pulmonary metastasis was assesed by means of tumor cell injection into the lateral tail vein of mice. Mice were injected intravenously with B16/BL6 melanoma(4×10^4) admixed with or without sulfated N-acetyl chitosan in PBS. The mice were killed on day 14 after tumor inoculation. The lung was fixed in Bouin's solution and the lung tumor colonies were counted under a dissection microscope.

In spontaneous pulmonary matastasis assay, mice were inoculated subcontaneously with B16/BL6(4.8×10^5) into the right hind footpad. The primary tumors were surgically removed on the day 21 after tumor innoculation. Sulfated N-acetyl chitosan was injected administered for several days before or after the amputation. Mice were killed on the day 14 after the number of lung colonies was counted(34).

Statistical analysis

The statistical significance of differences between the groups was determined by applying Student's t-test in the murine experimental and spontaneous metastasis model.

Results and Discussion

Fig. 1. shows the IR spectrum of chitosan, N-acetyl chitosan, and sulfated N-acetyl chitosan. Fig. 2 shows in the ^{13}C-nmr of sulfated N-acetyl chitosan. Sulfated group was detected by infrared absorptions at 1240~1250(S=O), and 800~1660cm (eq. C-O-S), N-acetyl group was detected at 1640~1660, and 1540~1560cm (C=O, and NH of N-acetyl). Because the raw material, chitosan, generally contains several percent of unhydrolyzed acetyl group, the bonds for unhydrolyzed acetyl group were also observed in the upper spectrum. Fig. 2. shows the^{13}C-nmr spectra of sulfated N-acetyl chitosan. ^{13}C signals

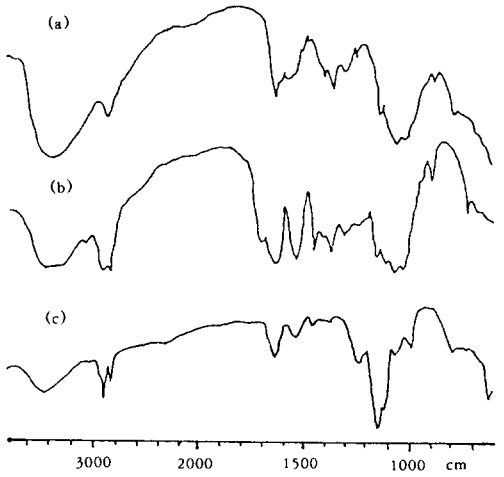

Fig. 1. Infrared spectra of chitosan(a), N-acetyl chitosan(b) and sulfated N-acetyl chitosan(c).

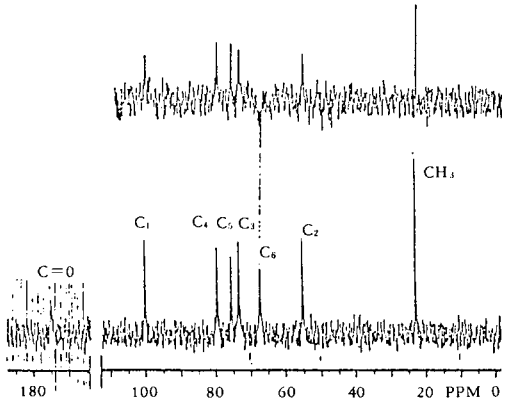

Fig. 2. ^{13}C-n. m. r. of sulfated N-acetyl chitosan.

of these compounds assigned as reported of chitosan. The CI and C4 signals in N-acetyl β-D-glucosamine and β-D-glucosamine HCl appear at 90.0~91.1ppm and 70.3~70.5ppm, respectively. These signals displace at 97.3~101ppm and 75.9~76.4ppm, due to β-D-glucosidation. The C6 signal appears at 67.5~67.3 ppm, indicating the O-sulfated of the hexosamine moiety. This deplacement agrees with the C6 signals at 67~68ppm in heparine(30), and at 69.33ppm in keratan sulfate(31) which has O-sulfate at C6 in the N-acetyl D-glucosamine moiety. The unsulfated C3 signal in N-acetyl β-D-glucosa-mine HCl, appears at 70.9ppm and 70.5ppm respectively. These data shows the formation of 3, 6-O-disulfated N-acetyl chitosan. The present method is applicable to analysis of the positions of substitution with O-sulfate at C6 and C3 in the hexosaminyl residue.

Table 1. Inhibition of tumor growth of B16/BL6 melanoma cells by i. v. injected sulfated N-acetyl chitosan(SNAC).

No. of mice	Doses (mg/kg)		Tumor Mean+SD(mm)	T/C(%)	p value
Control	6	–	836+20		
SNAC	6	20	476+12	43.1	<0.01
	6	40	360+27	56.9	<0.05
	6	60	268+35	67.9	<0.001
	6	80	188+43	77.6	<0.001
	6	100	187+18	77.7	<0.001

A) The ratios of treated group by control group. C57BL/6mice were administered i. v. injection with SNAC on days 1, 3, 6, 9 and 11 after B16/BL6 melanoma cells inoculation. Mice were killed on days 14 and were weight on day 14.

Inhibition of tumor cells

In this study, we synthesized a water soluble chitosan derivatives to observe the inhibitory effect the C57BL/6B mice bearing on B16/BL6 tumor cells.

Table 1. shows that i. v. injection of sulfated N-acetyl chitosan on given days 1, 3, 6, 9 and 11, inhibited markedly the growth of B16/BL6 melanoma cells in a dose of 100mg/kg. When the sulfated N-acetyl chitosan was administered at highest level of 100mg/kg, the tumor weight was reduced significantly comparing with the control group. Sulfated N-acetyl chitosan strongly inhibited the growth of tumor. Sulfated polysaccharide such as dextran sulfate(19), chitin heparinoid (29), chitosan(38) and carageenan (37) also inhibited the growth of tumor cells as similar this experiment.

Table 2. Effect of sulfated N-acetyl chitosan (SNAC) on experimental lung metastasis by i. v. injection on B16/BL6 melanoma cells.

No. of mice		Doses (mg/kg)	No. of lung metastasis on day 14 (Mean+SD)	p values[a]
Control	6	−	102+43	
SNAC	6	20	82+21	<0.001
	6	40	76+27	<0.001
	6	60	41+60	<0.005
	6	80	38+7	<0.001
	6	100	37+4	<0.001

C57BL/6 mice were admimistered i. v. injection with sulfated N−acetyl chitosan after B16/BL6 melanoma cells inoculation. Mice were killed on day 14 and tumor colonies in the lung were counted.

a) Compared with untreated control by Student's t test.

Inhibition of tumor cells metastasis

In the experimental lung metastasis, B16/BL6 melanoma cells were inoculatded into the C57BL/6 mice and then sulfated N-acetyl chitosan was injected I. V. to the C57BL/6 mice bearing B16/BL6. When tumor cells was treated with sulfated N-acetyl chitosan, the number of metastasis foci of B16/BL6 melanoma reduced in a dose dependent manner. Sulfated N-acetyl chitosan was dissolved in saline, and was injected immediately into C57BI/6B mice. Table 2 shows that sulfated N-acetyl chitosan inhibited lung metastasis in a dose dependent manner ranging from 20 to 100mg/kg. When sulfated N-acetyl chitosan was administered i.v. injection 3 times during 14days, the number of pulmonary metastatic foci were reduced at the dose dependent manner. Inhibition of metastasis was observed significantly when 100mg/kg of sulfated N-acetyl chitosan was injected into mice bearing B16/BL6 melanoma cells.

For the spontaneous lung metastasis, sulfated N-acetyl chitosan was administered in the two systems of the spontaneous lung metastasis induced by intra-footpad injection of B16/BL6 melanoma cells. The first administeration of sulfated N-acetyl chitosan injected to start on days 7 after tumor inoculation and the treatments were carried out 5 or 6 times at intervals of 2day or 3days. Primary tumors were surgically removed on day 21 and lung tumor colonies were counted for 14 days after tumor excision. Table 3 shows that two or three intervals of i. v. injection inhibited as maximum level of the lung tumor at the dose of 60mg/kg. In control mice whose primary tumors were resected on the days after i. v. inoculation, primary tumor size were observed no differences over the dose of sulfated N-acetyl chitosan on day 21. However, sulfated N-acetyl chitosan potently reduced the number of metastasis foci in a dose-dependent manner. The metastasis of the lung were inhibited by the injection of sulfated N-acetyl chitosan at doses of 60mg/kg on every 2days or 80mg/kg on every 3days. Sulfated N-acetyl chitosan also reduced the size of the metastatic foci. The intermittent i.v. injection of sulfated N-acetyl chitosan at 60mg/kg on

Table 3. Effect of sulfated N-acetyl chitosan[9] (SNAC) on spontaneous lung metastases by injection of B16/BL6 melanoma cells.

No.of mice		Doses (mg/kg)	Primary tumor size on day 21 (Mean+SD, mm)	NO. of lung metastasis on day 15 (Mean+SD)	p[a]
Experiment	1				
Control	6	−	102+43	94+12	
SNAC	6	40	10+3	63+20	
	6	60	10+1	25+22	<0.001
	6	80	10+1	26+11	<0.001
Experment	2				
control	6	−	10+2	90+35	
SNAC	6	40	11+1	50+8	<0.005
	6	60	10+2	18+1	<0.001
	6	80	10+2	17+3	<0.001

C57BL/6 mice were administered i. v. injection with SNAC on days 7, 9, 11, 13, 15, 17(experiment 1) or 7, 10, 13, 16, 19(experiment 2) intra-footpad injection(5×105) of melanoma cells. Primary tumor were surgically removed on day 21. Mice were killed on day 14 after tumor excision and tumor cells colonies in the lung were counted.

a) Compared with untreated control by Student's t test.

every 2 days reduced the number of metastic foci in the lung more effectively. Treatment of mice with sulfated N-acetyl chitosan prior to intravenous inoculation of tumor cells resulted in reduction of metastasis sulfated N-acetyl chitosan is a polyanionic compound. The negative charge on the surface of tumor cells and cultured enthotelium like cells was increased by sulfated N -acetyl chitosan. The polyanionic compounds such as chitin heparinoid(29), carageenan(37) and dextran sulfate(19) effectively inhibited the number of metastasis of tumor cells. Hagner and Norry(38) shows evidence of a direct binding of heparin to the surface of tumor cells. Nordling (39) tested the effect of dextran sulfate of the cells deformability, and the polymer made less deformable Ehrlich ascite tumor cells. The reason increased surface negatives charge by such as polyanionic compounds might be due to electric charges to the cell membrane. This phenomenon is possible charge material of the polyanionic sulfated N-acetyl chitosan resulted in the relative negative charge on the cells.

Effect of sulfated N-acetyl chitosan on tumor cells adhesion

Adhesion of metastatic tumor cells is considered an initial step of interaction with extracellular matrix. and basement membrane(1-4). The adhesive interaction between tumor cells and host cells or component of extracellular matrix plays an important role in the progress of tumor metastasis. Recently, some researches were carried out to inhibit the mechanism involved in tumor cell adhesion during the metastasis(14, 15). To investigate the mechanism of the reduction of tumor cells arrest in the lung, tumor cells adhesion were carried out to investigate the effect of sulfated N -acetyl chitosan on the adhesion of B16/BL6 cells to the coated with laminin. ^{125}I-labelled B16/BL6 cells were added to laminin coated wells in the presence or absence of sulfated N-acetyl chitosan. Table 4 shows that sulfated N-acetyl chitosan at various concentrations ranging from 50, 250, 500 or 700ug/ml specially inhibited

Table 4. B16/BL6 mice melanoma cells adhesion to laminin coated wells in the presence or absence of sulfated N-acetyl chitosan.

Treatment	Concentration (ug/ml)	Binding capacity (No. of cell bound+SD)	P[a)]
Control	–	4652+518	
SAC	50	3693+108	<0.01
	250	3074+116	<0.01
	500	2843+127(39%)	<0.01
	700	2837+108	<0.01

Labeled B16/BL6 tumor cells (2.0×104) were added to well precoated with 10ug of laminin with or without of 50, 250, 500, 700ug/ml of sulfated N-acetyl chitosan. Non -adhesive cells washed after 30 min incubation at 37 C and remaining adhesive cells counted. The values in parentheses represent percent inhibition.

a) Compared with untreated control by Student's t test.

tumor cells adhesion with laminin. Sulfated N-acetyl chitosan was able to inhibit the tumor cells adhesion at dose-dependant manner. 50ug/ml of sulfated N-acetyl chitosan inhibited the B16/BL6melanoma cells at the highest level of 39% which compared with that control group(Table 4). Cell adhesion molecules such as laminin have been found to contribute to cell adhesion(34). Such specific interaction is therefore a fundamental event in the metastatic process. The adhesion of tumor cells to the subendothelial matrix followed by endothelial cells retraction may be an important step in stable arrest of tumor in target tissue. Liotta, *at. al*(9) found that interaction of laminin on the tumor cell surface is important for sucessful lung colonization by tumor cells. The tumor cell adhesion is characterized initial arrest with tumor cell-endothelial contact and adhesion to endothelial matrix(35). In this study, sulfated N-acetyl chitosan shows inhibited B16/BL6 tumor cells adhesion to the endothelial matrix of laminin. These results suggested that sulfated N-acetyl chitosan inhibited tumor cell through the prevention of some steps in the invasive process including cell adhesion of tumor cells to endothelial matrix(3, 4, 14, 15). Chitin heparinoid(29) inhibited tumor cells adhesion, and dextran sulfate

(19) as a sulfated polysaccharide inhibited B16/BL6 melanoma tumor cells. Mutual adhesion between cells is reduced by an increase in surface negative electric charge because of an increased electrostatic repulsive force between them(40). Therefore, it is considered that mutual adhesiveness between tumor cells and endothelium cells can also be altered by an increased surface negative electric charge of both cells brought about by treatment of tumor cells with sulfated N-acetyl chitosan. This can then result in inhibition of lodgement of tumor cells with reduction in metastasis as a consequence.

요 약

키토산 유도체인 sulfated N-acetyl chitosan을 합성하여 이 화합물을 쥐에 이식한 B16/BL6 melanoma에 대하여 폐암전이의 억제효과를 조사하였다. 키토산의 황산유도체는 13C-n. m. r.에 의하여 확인한 결과 3, 6, O-disulfate 임을 알 수 있었다. Sulfated N-acetyl chitosan을 100mg/kg을 투여하였을 때 B16/BL6의 melanoma cells을 가장 효과적으로 억제하였고 종양 무게의 증식도 대조군에 비해 억제 되었다. 폐암의 전이에 있어서 B16/BL6에 sulfated N-acetyl chitosan을 I.V.로 주사하였을 때 B16/BL6의 melanoma의 전이 세포의 수는 자발적인 전이에 있어서도 용량 의존형(20~100mg/kg)에 따라 줄어들었다. B16/BL6을 접종한 후 sulfated N-acetyl chitosan을 I. V.로 투여했을 때 전이세포의 수는 현저히 감소하였다. Sulfated N-acetyl chitosan은 laminin의 종양세포에의 세포접착 능력을 부분적으로 억제하였다. 이와 같은 결과는 chitosan의 유도체인 sulfated N-acetyl chitosan이 세포접착 능력이 실험적으로 자발적인 암 전이 모델에서 효과적으로 작용하였다.

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References

1. P. S. Jones and S. S. De Clerk(1980), *Cancer Res.*, **40**, 3222-3230.
2. L. A. Liotta, S.Garbisa, and K. Tryggvason (1982), In : Liotta, L. A. and Hart, I. R. (eds.), *Tumor invassion and metastasis*, 319. The Hargu : Martinus Nighoff.
3. M. Makajima, D. R. P. N. Bolloni, and G. L. Nicolson(1987), *Cancer Res.*, **41**, 4876-4876.
4. G. L. Nicolson(1982), *Biochim. Biophys. Acta.*, **695**, 113-120.
5. G. L. Nicolson, M. Nakajima and T. Irimursa (1985), In invasion of vascular endothelium and organ tissue in vitro by B16/BL6 mel anoma vatients. In : K. V. honn, W. E. Power and B. F. Sloan(eds.), *Mechanisms of metastasis, Potential implications*, 275-289, The Hague : martinus Nijhoff.
6. I. R. Hart(1982), *Cancer Metastatsis Rev.*, 1, 5-16.
7. I. J. Fielder(1982), In cancer invasion and metastasis : Biological and therapeutic aspects(eds.), G. L. Nicolson and L. Milsa., *Raven Press, New York*, 5-26.
8. G. L. Nicolson (1987), *Cancer Res.*, **47**, 1473-1487.
9. L. A. Liota, C. V. Rao and S. H. Barsky (1983), *Lab. Invest.*, **49**, 639-649.
10. S. Kaspathlan and E. pearlstein(1984), In : Hemostasis mechanism and metastasis(eds.), K. V. Honn and B. F. Sloane, Martinus Nijhoff, Boston, 139-169.
11. J. B. mcCarthy and L. T. Furcht(1984), *J. Cell. Biol.*, **98**, 1474-1480.
12. V. P. Teranova, E. S. Hujanon and G. R. Martin(1986), *J. Natl. Cancer Inst.*, **77**, 311-316.
13. Y. Hara, M. Steiner and M. G. Baldini(1986), *Cancer Res.*, **40**, 1217-1220.
14. P. Hilgard, H. heller and C. G. Schmidt and Z. Krebsforsh(1976). *Z. Krebsforsch*, **86**, 243-250.
15. E. Tsubura, T. Yamashita, M.Kobayashi, Y. Higuchi and J. Isobe(1977), *Gann Monogra. Cancer Res.*, **20**, 147-153.
16. T. Imura, M, Nakajima and G. L. Nicolson (1986), *Biochemistry*, **25**, 5322-5337.

17. J. Irida, P. N. Skubitz, L. T. Furcht, E. A. Wayner, and J. B. McCarthy(1992), *J. Cell Biology,* **118**, 431-452.
18. S. L. Drake, D. J. Klein, D. J. mickelson, T. R. Oegema, L. T. Furcht and J.B. McCarthy (1992), *J. Cell Biology,* **117**, 1331-1348.
19. K. Suemasu(1977), *Gann monograph cancer Res.,* **21**, 150-161.
20. D. Knorr(1984), *Food Technol.,* **85**, 20-32.
21. R. A. A. Muzzarelli, (eds.)(1978), In chitin, Pergamon Press, Oxford, UK, 139.
22. S. Miyazaki, I. Ishii, S. Nishimura, N. nishi, S. Totura and I. Azuma(1989), *Japn. J. Cancer Res.,* **80**, 866-877.
23. Y. Sawayanagi, N. Nambu, and N. Nagai (1982), *Chem. Pharm. Bull.,* **30**, 4213-4215.
24. K, Watanabe, I. Saiki, Y. Matsumoto, I. Azuma, Y. Uraki, Y. Miura and S. Tokura (1992), *Carbohydr. Polym.,* **17**, 29-37.
25. K. Suzuki, Y. Okawa, S. Hashimoto, S. Suzuki, and M. Suauki(1984), *Microbiol. immunol.,* **29**, 234-239.
26. B. Alarcon, J. C. Lacal, J.M. Fernandes-Sousa and L. Carrasco(1984), *Antivral Res.,* **4s**, 234-244
27. S. Suzuki, Y. Okawa, Y. Okura., K. Hashimoto, and M. Suzuki(1982), *Proc. 2nd Interaction Cof. Chitin and Chitosan,* S. Hirano and S. Tokura(eds.) *Japn. Soc. Chiton/ Chitosan,* 210
28. J. Murata, I. Saiki, S. nishimura, N. Nishi, S. Tokura and I. Azuma(1989), *Japn. J. Cancer Res.,* **80**, 866-872.
29. J. Murata, J. Saiki, M. Nakajima, S. Tokura and I. Ajuma(1989), *Cancer Res.,* **50**, 3631-3653.
30. S. Hirano and J. Kinugawa(1986), *Carbohydr. Res.,* **295**, 150-158.
31. R. G. Schweigh(1972), *Carbohydr. Res.,* **21**, 219-230.
32. A. Albi, Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aronson, J. M. Kozlowski, and R. N. McEman(1987), *Cancer Res.,* **47**, 3239-3245.
33. I. Saiki, J. Murata, N. Nishi, R. Ogawa, K. Sugimura., S. Tokura and I. Azuma(1989), *Japn. Cancer Res.,* **49**, 3815-3822.
34. J. Folkman, R. Langer, R. J. Lingardt, C. Handenschild and S. Taylor,(1977), *Science* (Washington DC), **197**, 893-896.
35. J. Murata J. Saiki, S. Nishimura, N. Nishi (1989), *Int. J. Biol. Macromol.,* **11**, 97-104.
36. B. H. Ryu(1992), *J. Kor. Food Nutr.* **21**(2), 154-162
37. W.J. Kort, A. M. Bijma and I. M. hekking (1970), *Clin. Exper. Metastases,* **7**, 117-126.
38. B. Hagmar and ak. Norrby(1970), *Intl. J. Cancer,* **5**, 72-86.
39. S. A. Nordling(1967), *Acta. Pathol. Microbiol., Scand., Suppl.,* **192**, 47-53.
40. M. Abercrombie and E. J. Ambrose(1962), *Cancer Res.,* **22**, 525-532.