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Marked Heterogeneity of Aromatase Activity in Human Malignant Melanoma Tissue

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Abstract—*The prognosis from human malignant melanoma varies according to sex and to multiple histologic, biologic and cell kinetic parameters. Thus melanomas exhibit a major degree of heterogeneity in their biologic properties and further characterization of their biochemical heterogeneity should yield important information. The present study sought to demonstrate the activity of a biochemical marker of estrogen synthesis, the aromatase enzyme, in melanoma tissue and to determine its range of activity. Initially, we validated a highly sensitive radiometric assay for aromatase by comparing it with a direct product isolation method. We detected production of* 417 pmol/g *protein/*h *of estrone and* 37.3 pmol/g *protein/*h *of estradiol by direct product isolation in a human melanoma and* 398 pmol *estrone/*g *protein/*h *by the radiometric assay. The activity present was blocked by similar amounts of the aromatase inhibitor, aminoglutethimide, as were necessary to block placental, breast cancer, and rat brain aromatase activity. We then assayed aromatase radiometrically in* 19 *human melanomas and found measurable activity ranging from* 9 *to* 398 pmol *estrone/*g *protein/*h *in* 15 *tissues. No relationship with the patient's age or sex was demonstrated. The activity exceeded by* 2*-fold that previously detected in* 49/61 *human breast cancers. This study identified a marker enzyme in melanoma tissue which varied by* 40*-fold among human tumors. Correlation of aromatase activity with prognosis and response to various types of therapy is now necessary to establish the biologic relevance of this finding.*

INTRODUCTION

THE PROGNOSIS of patients with malignant melanomas varies widely and correlates independently with the sex of the patient and the site of the lesion, tumor thickness, ulceration, mitotic rate, type of dominant invasive tumor cell and with lymphocytic infiltration [1–5]. These clinical observations suggest that melanomas are heterogeneous in their biologic properties and that host responses to the tumor vary. Based upon these findings, further characterization of the biochemical properties of melanoma tissue should yield important information. Properties which could relate to the marked difference in prognosis between men and women would be particularly relevant [2].

As part of a systematic study of potentially endocrine-responsive human tumors, we detected the presence of the enzyme aromatase in a human melanoma. This enzyme catalyzes the conversion of androgens to estrogens in peripheral tissues and in malignancies such as breast cancer [6–8]. We considered this preliminary finding potentially important since animal and human data have suggested that melanomas are heterogeneous regarding their responsiveness to estrogen [9–16]. The hamster melanoma model HM-1 contains estrogen receptors, grows more rapidly in female animals and can be inhibited by the antiestrogen, nafoxidine [17]. The rate of growth of a subset of human melanomas may be altered by pregnancy [9], parturition [10], and onset of menses [11]. Responses to hypophysectomy, estrogens, anti-estrogens, progestins and androgens have been reported in human melanoma patients, but only in a small percentage [13–16].

Based upon these clinical and biological considerations, we considered it pertinent to study the aromatase-mediated production of estrogen by these tumors in detail. The goals of this study were to convincingly establish the presence of the aromatase enzyme in melanoma tissue and to quantitate its activity. The present report indicates that human melanomas contain highly variable (i.e. 40-fold different) activities of this enzyme. This high degree of variability is potentially of great interest and further studies are now required to correlate

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aromatase activity with the biological properties of the tumor, with patient prognosis, and with response to therapy.

MATERIALS AND METHODS

Tissue preparation

Malignant melanoma tissues, obtained within 30 min of surgical excision, were frozen in liquid nitrogen, pulverized, and stored at −70°C until use. Portions of tissue were examined histologically to confirm the presence of malignant melanoma and contiguous portions were used for the aromatase analysis.

[^3H]Water radiometric assay

Measurement of estrone production from 1β-[^{3}H]androstenedione (specific activity of 40–60 Ci/mmol) utilized the radiometric [^{3}H]water (^3H_2O) assay of Thompson and Siiteri [18] as modified slightly by our group [8]. Tissue homogenates representing the equivalent of 25 mg wet wt of tissue were incubated with 56.2 pmol [^{3}H]androstenedione per tube. All tissues were run in duplicate. Minimum counts in positive samples were at least 30 cpm/tube. We have validated this method for determining aromatase activity in brain [19], placenta [20] and breast carcinona tissue [8] by comparing the results obtained with those from a direct product isolation assay. The precise methodologic details of our assay procedure have been published previously [8]. Procedural steps for the direct product isolation method to validate the radiometric technique have also been previously described [8].

The slight modifications used for melanoma tissue in this study are described under Results. The 7α-[^{3}H]androstenedione (55.8 pmol/tube added with specific activity of 0.030 mCi/pmol) used for these studies was custom-synthesized by the New England Nuclear Corporation under the direction of Dr. Cabell.

With the radiometric aromatase assay, we have found ^3H_2O release to be linear with respect to time and amount of enzyme, to be saturable, and to be inhibited by specific aromatase blockers shown to be effective in human breast cancer, placental tissue, and in rat brain [8, 19, 20]. Slightly modified versions of this radiometric assay are now standard for quantitation of aromatase activity in a variety of tissues [18, 19, 21, 22].

Receptor determinations

Estrogen receptors (ER) were measured using the method of McGuire *et al.* [23]. Progesterone receptor (PgR) measurements utilized the method of Feil *et al.* [24]. Quality control powders obtained from Dr. James Wittliff at the University of Louisville, KY are routinely run for ER and PgR and fall within the mean and standard deviation range of participating laboratories.

RESULTS

Validation of radiometric assay specificity

Although the radiometric ^3H_2O aromatase assay is used widely [14–18], we considered the possibility that melanoma tissue might have unique properties which could invalidate this indirect method. Zava and Goldhirsch [25] demonstrated that melanoma tissues contain tyrosinase and other enzymes which can release [^{3}H]water from labeled estradiol by nonaromatase-catalyzed reactions. For this reason, we utilized two methods to further validate the radiometric assay: product isolation and specific enzyme inhibition.

Product isolation

In aliquots of 1000 ***g*** supernatants from homogenates of human melanoma, aromatase activity was assayed simultaneously by isolation of 7α-[^{3}H]estrone and estradiol products formed from 7α-[^{3}H]androstenedione and by the ^3H_2O release assay. Both methods used similar concentrations of substrate (i.e. 53.1 pmol for the ^3H_2O assay and 72 pmol for product isolation). These concentrations are nearly 10 times the usual K_m for aromatase and thus are believed to saturate the enzyme. The product isolation assay was modified slightly from our previous method [8] with respect to the chromatographic systems used as outlined in Table 1. Constant ^{3}H:^{14}C ratios were present after the first chromatographic step (Table 1). A second experiment demonstrated that the loss of product by further estrogen metabolism during incubation in the direct assay was minimal. In tubes containing tissue, 70.2% of radiolabeled estrone was recovered as estrone and 3.3% as estradiol when compared to tubes not containing tissue; similarly, with addition of estradiol 72.3% was recovered as estradiol and 3.2% as estrone.

Using this modified technique, we detected 417 pmol of estrone and 37.3 pmol of estradiol/g protein/h of radiochemically pure (i.e. constant ^{3}H:^{14}C ratios) estrogen products of aromatase. By comparison, the radiometric assay yielded similar results (398 pmol estrone/g protein/h) and clearly did not overestimate aromatase activity.

Enzyme inhibition

To further establish that the [^{3}H] water assay specifically detected aromatase, aminoglutethimide, a potent type II aromatase inhibitor [20, 26] was added to incubations at various concentrations (Fig. 1). Fifty per cent inhibition of activity was produced by a concentration of 12 μM which had been shown to produce similar inhibition of aromatase activity in human placenta [20], breast cancer

Table 1. Purification of estrone and estradiol from androstenedione

Chromatographic step	Estrone		Estradiol	
	^3H (cpm)	^3H:^{14}C	^3H (cpm)	^3H:^{14}C
TLC [ether–methylene chloride (10:90)]	15487	68.0	7214	35.8
TLC [methanol–methylene chloride (5:95)]	7373	22.1	591	2.10
TLC [chloroform–ethyl acetate (80:20)]	3060	20.9	314	2.10
Acetylation, followed by				
TLC [ether–methylene chloride (4:96)]	5808	21.8	443	2.11

Counts are corrected for background and ^{14}C spillover, but not for aliquot size.

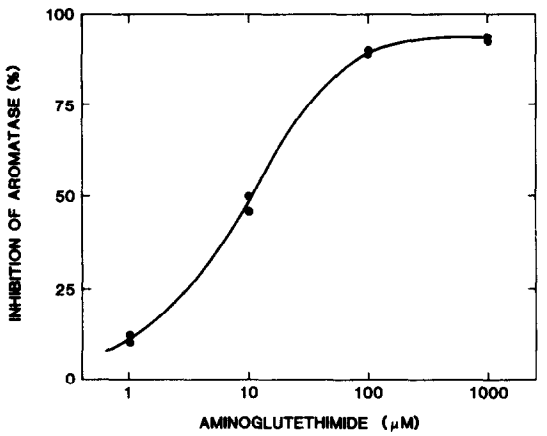

Fig. 1. Inhibition of aromatase with aminoglutethimide in concentrations from 1–1000 μM. Results are expressed as percentage inhibition of aromatase activity.

tissue [8] and in rat brain [19]. Nearly complete inhibition was observed at the highest concentration of aminoglutethimide, a finding which lends credence to the specificity of the ^3H_2O assay.

Quantitation of aromatase activity

Having validated the radiometric assay for melanoma tissue, we measured aromatase activity in 19 human tumors. Fifteen contained detectable levels (i.e. >5 pmol/g protein/h) ranging from 9.3 to 398 pmol/g protein/h with a median level of 26 pmol/g protein/h. No apparent relationship existed between the levels obtained vs. patient age or sex (Table 2). By contrast, in studies utilizing the B16 murine melanoma, detectable aromatase levels were found in male mice (10–102 pmol/g protein/h, $n = 4$) but not in females (<5 pmol/g protein/h, $n = 3$).

DISCUSSION

The present study questioned whether human malignant melanomas contain aromatase activity and whether the levels of this estrogen synthesizing enzyme are sufficiently variable to serve as a marker with which to subclassify biological variants of the tumor. Our data from radiometric and product isolation assays as well as use of specific enzyme inhibitors convincingly demonstrate the presence of

Table 2. Aromatase levels in patients with melanomas

Patient	Sex	Age	Estrogen receptor	Progesterone receptor	Aromatase (pmol/g protein/h)
1	M	50	<10	<10	182
2	F	37	<10	<10	61
3	M	79	ND*	ND	130
4	M	52	ND	ND	63
5	M	30	<10	<10	75
6	M	79	<10	<10	22
7	M	28	<10	<10	26
8	M	55	<10	<10	<5
9	F	78	ND	ND	82
10	M	43	ND	ND	17
11	F	79	ND	ND	9
12	M	26	<10	<10	12
13	F	65	<10	<10	30
14	F	49	ND	ND	398
15	M	51	<10	<10	9
16	F	61	<10	<10	<5
17	M	69	<10	<10	40
18	F	31	29	<10	<5
19	F	ND	<10	<10	<5

*Not determined.

aromatase activity in melanoma tissue. Levels of detectable enzymatic activity varied 40-fold among tumors with a lowest level of 9.3 and highest of 398 pmol/g protein/h of estrogen formed from androstenedione. In addition, five tumors contained no measurable activity. These widely varying levels document a high level of heterogeneity of melanoma tissue in its ability to synthesize estradiol from androgen precursors *in situ*. The results of this study suggest a potential mechanism whereby the degree of tumor heterogeneity could serve to alter the interplay between tumor and host. Notably, estrogen-dependent melanomas containing high levels of aromatase activity and producing high local concentrations of estradiol might display different growth characteristics from tumors lacking these properties. At present, this construct is hypothetical and requires testing in a large number of patients. Nonetheless, the presence of aromatase provides a measurable biochemical parameter with which to identify subsets of human melanomas.

The degree of hormone dependence of malignant melanomas is not fully understood at present. The hamster melanoma cell line HM-1 contains estrogen receptors, grows more rapidly in female animals and is inhibited by antiestrogen therapy [17]. Studies using a variety of biochemical techniques have demonstrated estrogen receptors in human melanomas [27–32]. Another means of establishing the hormonal dependence of human melanoma tissue is to demonstrate changes in growth characteristics in response to alterations in the hormonal milieu. Preliminary clinical observations suggest altered melanoma growth rates in pregnant patients with stage II melanoma [9] and objective tumor regressions in 5–20% of patients after administration of tamoxifen, diethylstilbesterol or medroxyprogesterone acetate [13–16]. A conservative approach to analysis of these cases and the available receptor data suggest to us that a subset of tumors are hormone dependent.

An interpretative problem in the available data is that results regarding estrogen receptors in human melanomas [28–32] have been recently questioned [25]. Tyrosinase, and perhaps other enzymes present in melanomas, can artifactually increase apparent receptor levels. By enzymatically removing ^{3}H from ligand labeled at position two and catalyzing formation of non-dextran-coated charcoal-bound metabolites, Scatchard plots are altered in a fashion closely mimicking competition for ligand by receptor [25]. Use of hydroxylapatite to precipitate receptor, tracer labeled at position one only or addition of excess L-Dopa or tyrosine to block tyrosinase should markedly reduce this artifact [25].

With use of such methods, estrogen receptors have still been found in human melanomas [28, 32]. We, on the other hand, detected the presence of estrogen receptor in only one of 19 patients in this study. Our estrogen receptor assay was designed to measure levels above 10 FM/mg cytosol protein. We have not established the validity of values below 10 FM/mg cytosol protein and consider such tumors receptor negative. However, two laboratories recently demonstrated, both by ligand binding and monoclonal antibody ELISA techniques, that the majority of ER+ human melanomas contain only 1–10 FM/mg cytosol protein in most instances [33, 34]. These investigative groups found that 56 and 43% of melanomas were ER+ using their methodology [33, 34]. Furthermore, Walker *et al.* [34] found that low ER+ melanomas were biologically different from absolutely ER− tumors. For example, the disease free survivals and absolute survivals were longer in the low ER+ (i.e. 1–10 FM/mg cytosol protein) than in the absolutely negative groups [34]. Based upon these data, it is likely that our assay was insufficiently sensitive to detect low ER+ values and that a larger fraction of the melanomas studied would probably contain ER if measured with more sensitive techniques. Consequently, it is likely that aromatase + tumors might also contain ER, if studied under the appropriate conditions.

Aromatase activity has now been detected in several human tissues including liver [35], muscle [36], stroma from fat tissue [37], pubic skin fibroblasts [38], brain [19], testes [39], and ovary [6]. In this study, we also demonstrated aromatase activity in malignant melanoma tissue. The biologic importance of extragonadal and extra-adrenal aromatase has been well established since all estrogen formed by postmenopausal women originates from the peripheral conversion of androgens to estrogens [7]. Interest in aromatase in peripheral tissues, particularly in fat cells, has been intensified by the recent observation that its activity can be substantially increased *in vitro* by the addition of glucocorticoids [40]. The importance of aromatase in producing *in situ* estrogenic effects is suggested by studies in rat hypothalamus. In this tissue, androgens must be converted locally to estrogens before exerting masculinizing effects on these tissues [41, 42].

With the perspective gained from these observations, it appears that the biologic relevance of local aromatase activity in cancer tissue and particularly in melanomas is an important issue. Several facets remain to be resolved. The specific cells within heterogeneous tumors wich contain aromatase are not known. This could be important since one cell type could produce estrogen which would then affect surrounding cells in a paracrine fashion. Alternately, an autocrine mechanism could predominate whereby the cell of estrogen production would respond itself to the high intracellular

estrogen concentrations. If the autocrine mechanism were correct, high local concentrations of aromatase in certain cell types would be important. Under these conditions the overall tissue concentrations of enzyme might be low as a function of dilution with cells not containing aromatase. This phenomenon appears to be present in rat hypothalamus where whole tissue preparations contain relatively low concentrations of aromatase but isolated nuclei contain up to 50 times higher levels. These concepts regarding local intracellular estradiol pools are important since questions have been raised previously about the potential biological significance of aromatase activity in tissues where substantially lower activities are found than that in the placenta [43].

In summary, we have demonstrated 40-fold differences in the levels of aromatase among human melanoma tissues. The biologic relevance of this high degree of tumor heterogeneity and of the host response to it is of potential interest. However, correlation with histologic types, patient prognosis, and response to various hormonal and chemotherapies must now be established.

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REFERENCES

1. Sondergaard K, Schou G. Therapeutic and clinico-pathological factors in the survival of 1469 patients with primary cutaneous malignant melanoma in clinical stage I. *Virchows Arch (Pathol Anat)* 1985, **408**, 249.
2. O'Doherty CJ, Prescott RJ, White H, McIntyre M, Hunter JAA. Sex differences in presentation of cutaneous malignant melanoma and in survival from stage I disease. *Cancer* 1986, **58**, 788–792.
3. Clark WH, Elder DE, van Horne M. The biologic forms of malignant melanoma. *Human Pathol* 1986, **17**, 443–450.
4. Seigler HF. Investigative studies in the diagnosis and treatment of melanoma. *Invest Radiol* 1986, **21**, 596–600.
5. Welvaart K, Hermans J, Zwaveling A, Ruiter DJ. Prognosis and surgical treatment of patients with stage I melanomas of the skin: a retrospective analysis of 211 patients. *J Surg Oncol* 1986, **31**, 79–86.
6. Siiteri PK, MacDonald PC. Role of extraglandular estrogens in human endocrinology. In: Greep RO, Astwood EB, eds. *Handbook of Physiology*. American Physiological Soc., 1973, Section 7, vol. II, Part 1, 615.
7. Grodin JM, Siiteri PK, MacDonald PC. Source of estrogen production in postmenopausal women. *J Clin Endocrinol Metab* 1973, **36**, 207–214.
8. Tilson-Mallett N, Santner SJ, Feil PD, Santen RJ. Biological significance of aromatase activity in human breast tumors. *J Clin Endocrinol Metab* 1983, **57**, 1125–1128.
9. Shiu MH, Schottenfeld D, MacLean B, Fortner JG. Adverse effect of pregnancy on melanoma. *Cancer* 1976, **37**, 181–187.
10. Shaw HM, Milton GW, Farago G, McCarthy WH. Endocrine influences on survival from malignant melanoma. *Cancer* 1978, **42**, 669–677.
11. Meyskens FL Jr. The endocrinology of malignant melanoma. *Rev Endocrinol Rel Cancer* 1981, **9**, 5.
12. Lee JH. Proceedings of the 11th International Pigment Cell Conference, 1980, Abst 124.
13. Bodenham DC, Hale B. Malignant melanoma. In: Stoll BA, ed. *Endocrine Therapy and Malignant Disease*. London, WB Saunders, 1972, 377.
14. Neifeld JP, Lippman ME. Steroid hormone receptor and melanoma. *J Invest Dermatol* 1979, **74**, 379–381.
15. Beretta G, Tabiadon D, Fossati P. First International Congress on Hormones and Cancer. *Cancer Treat Rep* 1979, **63**, 1200 (Abst 285).
16. Nesbit RA, Woods RL, Tattersall MHN *et al.* Tamoxifen in malignant melanoma (letter). *New Engl J Med* 1979, **301**, 1241–1242.
17. Schleicher RL, Hitselberger MH, Beattie CW. Inhibition of hamster melanoma growth by estrogen. *Cancer Res* 1987, **47**, 453–458.
18. Thompson EA Jr, Siiteri PK. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J Biol Chem* 1974, **249**, 5364–5372.
19. Weisz J, Brown BL, Ward IL. Maternal stress decreases steroid aromatase activity in brains of male and female rat fetuses. *Neuroendocrinology* 1982, **35**, 374–379.
20. Santen RJ, Santner SJ, Tilson-Mallet N, Rosen HR, Samojlik E, Veldhuis JD. *In vivo* and *in vitro* pharmacologic studies of aminoglutethimide as an aromatase inhibitor. *Cancer Res* 1982 (Suppl), **42**, 3353S.
21. Johnston JO, Wright CL, Metcalf BW. Biochemical and endocrine properties of a mechanism-based inhibitor of aromatase. *Endocrinology* 1984, **115**, 776–785.
22. Brodie A, Santen RJ. Aromatase in breast cancer and the role of aminoglutethimide and

other aromatase inhibitors. In: *Critical Reviews in Oncology/Hematology*. Boca Raton, CRC Press, 1987, vol 5, 361.
23. McGuire WL, De La Garza M, Chamness GC. Evaluation of estrogen receptor assays in human breast cancer tissue. *Cancer Res* 1977, **37**, 637–639.
24. Feil PD, Klase JC, Margets MJ. Use of medroxyprogesterone assay to measure cytosol progestin receptors in human breast cancer. 61st Annual Meeting of the Endocrine Society, Anaheim, CA, 1979, p. 150 (Abst).
25. Zava DT, Goldhirsch A. Estrogen receptor in malignant melanoma. Fact or artifact. *Eur J Cancer Clin Oncol* 1983, **19**, 1151.
26. Graves PE, Salhanick HA. Stereoselective inhibition of aromatase by enantiomers of aminoglutethimide. *Endocrinology* 1979, **105**, 52–57.
27. Chaudhuri PK, Walker MJ, Briele HA, Beattie CW, DasGupta TK. Incidence of estrogen receptor in benign nevi, and human malignant melanoma. *J Am Med Assoc* 1980, **244**, 791–793.
28. Rumke P, Persijn JP, Korsten CB. Oestrogen and androgen receptors in melanoma. *Br J Cancer* 1980, **41**, 652–656.
29. Stedman KE, Moore GE, Morgan RT. Estrogen receptor proteins in diverse human tumors. *Arch Surg* 1980, **115**, 244–248.
30. Creagan ET, Ingle JN, Green SJ, Ahmann DL, Jaing N-S. Phase II study of tamoxifen in patients with disseminated malignant melanoma. *Cancer Treat Rep* 1980, **64**, 199–201.
31. Kokoschka EM, Spona J, Bieglmayer C, Schmidt J. First International Congress on Hormones and Cancer. *Cancer Treat Rep* 1979, **63**, 1200 (Abst 284).
32. McCarty KS Jr, Wortman J, Stowers S, Lubahn DB, McCarty KS Sr, Seigler HF. Sex steroid receptor analysis in human melanoma. *Cancer* 1980, **46**, 1463–1470.
33. Ferno M, Borg A, Ingvar C, Jonsson P-E. Estrogen receptor and binding site for estramustine in metastatic malignant melanoma. *Anticancer Res* 1987, **7**, 741–744.
34. Walker MJ, Beattie CW, Patel MK, Ronan SM, Das Gupta TK. Estrogen receptor in malignant melanoma. *J Clin Oncol* 1987, **5**, 1256–1261.
35. Smuk M, Schwers J. Aromatization of androstenedione by human adult liver *in vitro*. *J Clin Endocrinol Metab* 1977, **45**, 1009–1012.
36. Longcope C, Pratt JH, Schneider SH, Fineberg SE. Aromatization of androgens by muscle and adipose tissue *in vitro*. *J Clin Endocrinol Metab* 1978, **46**, 146–152.
37. Cleland WH, Mendelson CR, Simpson ER. Aromatase activity of membrane fractions of human adipose tissue stromal cells and adipocytes. *Endocrinology* 1983, **113**, 2155–2160.
38. Bulard J, Mowszowicz I, Strouve-Vallet C, Schaison G. Augmentation de l'activité aromatase dans les fibroblastes de peau de sujets porteurs de gynécomastie. *Ann Endocrinol* 1985, **46**, 148 (Abst 48).
39. Rommerts FFG, deJong FH, Brinkmann AO, VanderMolen HJ. Development and cellular localization of rat testicular aromatase activity. *J Reprod Fertil* 1982, **65**, 281–288.
40. Mendelson CR, Cleland WH, Smith ME, Simpson ER. Regulation of aromatase activity of stromal cells derived from human adipose tissue. *Endocrinology* 1982, **111**, 1077–1085.
41. Pardridge WM, Gorski RA, Lippe B, Green R. Androgens and sexual behavior. *Ann Intern Med* 1982, **96**, 488–501.
42. Roselli CE, Horton LE, Resko JA. Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology* 1985, **117**, 2471–2477.
43. Bradlow HL. A reassessment of the role of breast tumor aromatization. *Cancer Res* 1982 (Suppl) **42**, 3382S.