表 11 おもな予後推定の指標

1, Prognostic Nutritional Index (PNI)[2]
PNI(%)=158-(16.6×ALB)-(0.78×TSF)-(0.20×TFN)-(5.8×DH)
ALB: 血清アルブミン(g/100ml)
TSF: 上腕三頭筋皮下脂肪厚(mm)
TFN: 血清トランスフェリン(mg/100ml)
DH: 遅延型皮膚過敏反応
0: 反応なし
1: ＜5mmの硬結
2: ≧5mmの硬結
PNI≧50%: high risk
PNI=40-49%: intermediate risk
PNI＜40%: low risk

2, 胃癌患者に対する栄養学的手術危険指数 (Nutritional surgical risk index; NRI)[3]
NRI=10.7ALB+0.0039Lymph. Count+0.11Zn-0.044AGE
ALB: 血清アルブミン(g/dl)
Lymph. Count: 末梢リンパ球数(/mm^3)
Zn: 血清亜鉛(μg/dl)
AGE: 年齢(years)
NRI≦55: high risk
NRI＞60: low risk

3, 食道癌患者に対する栄養評価指数 (nutritional assessment index; NAI)[4]
NAI=2.64AC+0.6PA+3.76RBP+0.017PPD-53.8
AC: 上腕周囲(cm)
PA: プレアルブミン(mg/dl)
RBP: レチノール結合蛋白(mg/dl)
PPD: PPD皮内反応〔直径×短径〕(mm^3)
NAI≧60: good
60＞NAI≧40: intermediate
40＞NAI: poor

4, StageⅣ・Ⅴ (Ⅴは大腸癌) 消化器癌患者に対するPNI[1]
PNI=10Alb.+0.008Lymph. C.
Alb.: 血清アルブミン(g/dl)
Lymph. C.: 末梢総リンパ球数(/mm^3)
PNI45以上: 手術可能
45＞PNI＞40: 注意一危険
PNI40以下: 切除・吻合禁忌

5, 消化器外科患者に対するPNIr[5]
PNIr=-0.147×(体重減少率)+0.046×(体重身長比)
+0.010×(三頭筋部皮厚比)+0.051×(ヘパプラスチンテスト)
PNIr10以上: 合併症なし
PNIr5-10: 移行帯
PNIr5未満: 合併症発生

の式”と呼ぶのが妥当と思われる．しかし多くの論文でWHOの式と呼ばれているため，混乱を避けるため本稿では“いわゆるWHOの式”（表3）としたことを付け加える．

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Clinical Significance of PNI in Infants with Cardiac Surgery

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The aim of our study was to (1) examine whether PNI, which was originally designed for adults with colon cancers, can be available for infants who received cardiac surgeries, and (2) to set the proper criterion of PNI. PNI was calculated respectively with laboratory data before the operation and was examined clinical significance on outcome index of length of stay in ICU (LS-ICU) postoperatively. When designating the cut-off value of PNI at 55, LS-ICU of the low PNI group was statistically and significantly longer than that of the high PNI group. Therefore when designating the cut-off value of PNI at 55 for infants with 0-18 month, PNI patients could be expected to have clinical outcome of LS-ICU. There is the possibility that PNI is a useful clinical index to predict the period of postoperative recovery for cardiac surgical infants.

PNI, infant, outcome index, cardiac surgery, length of stay in ICU

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原　著

乳幼児の術前の身体指標は術後のアウトカム指標となり得るか —心疾患症例における検討—

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松岡　美緒[3)]　雨海　照祥[1)]

栄養状態（H/A, W/H），病態の重症度（PRISM Ⅲ）および手術侵襲の大きさが，アウトカム指標（COI）としての意義をもつのかを検討した．対象は月齢18カ月以下の心臓手術患児とした．H/A, W/H, PRISM Ⅲを整数値で1ずつ区切り，その境界（カットオフ）値未満とそれ以上の2群間，手術侵襲の大きさにより分類した2群間でCOIを比較検討した．COIには，ICU在室日数，術後在院日数，人工呼吸器管理期間の3指標を用いた．その結果今回我々が対象とした月齢18カ月以下の心臓手術症例においては，(1) H/Aが低値となるのに生後3カ月を要する，(2) 術前の栄養指標はCOIとならない，(3) PRISM Ⅲおよび手術侵襲の大きさがCOIとなる，ことが判明した．

乳幼児，Waterlow分類，アウトカム指標，PRISM Ⅲ，アットリスク症例

はじめに

乳幼児の心疾患症例において，予後推定栄養指数（PNI）が，アウトカム指標としての術後ICU在室日数を予測できるアウトカム指標となり得ることを報告した[1)]．一方，小児の栄養アセスメントの指標として頻用されるWaterlowの分類[2,3)]が，はたして予後判定あるいは主に有害事象の発生率などで計測されるアウトカムの予測指標として利用可能なのか，いまだ検討されていない．

そこで今回我々は，栄養アセスメント指標が臨床的アウトカム指標（Clinical Outcome Indicator：COI）となり得るかを明らかにする目的で，月齢0～18カ月の心臓手術を施行された患児を対象として，Waterlow分類が，COIとなり得るか否かを検討した．さらに術後アウトカムに影響を与えると考えられる病態の重症度および手術侵襲の大きさ（侵襲度）についても同様に，COIとしての妥当性の有無を検討した．

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対象と方法

1. 対象

2007年8月から2008年4月までの9カ月間に，兵庫県立こども病院で心臓手術を施行された，月齢18カ月以下の患児25例（男児18例，女児7例）を対象とした（表1）．

表1　対象の身体プロフィール（mean±SD）

対象の臨床プロフィール	
n（男児/女児）	25（18/7）
月齢（カ月）	5.2±6.2
身長（cm）	59.4±14.0
体重（kg）	5.3±2.1
身長年齢比（H/A，%）	99.5±10.2
体重身長比（W/H，%）	88.0±12.3

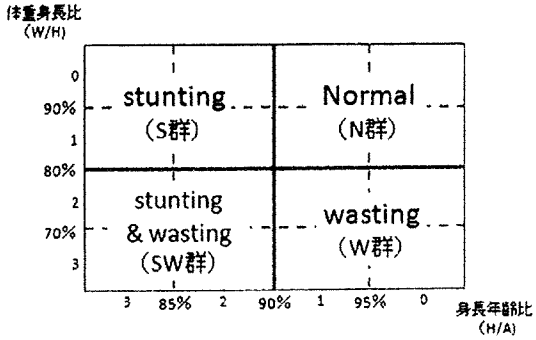

図1　Waterlow分類による小児の栄養障害のタイプ分け

2. 方法

術後COIに影響を及ぼす可能性のある因子として術前の栄養状態および病態の重症度に注目し，これらがCOIとなり得るかを検討した．ここでCOIの指標としては①ICU在室日数，②術後在院日数，③人工呼吸器管理期間，の3指標を用いた．

1. 方法1　術前の栄養状態がCOIの影響因子となり得るか否かの検討

(1) 術前の身長年齢比（Height for Age：H/A）を1ずつ整数値で区切り，その境界（以下，カットオフ）値未満とそれ以上の2群間でのCOIの有意差の有無を検討した．

(2) 慢性的栄養障害の指標とされるH/Aが出生後に低下するまでの期間を検討する目的で，月齢を1カ月毎に区分し，その月齢未満とそれ以上の2群間のH/Aの比較検討を行った．

(3) 上の方法1 (2) で求められたH/Aが低値となるのに要する期間以降の月齢の症例に限って，方法1 (1) 同様にカットオフ値未満とそれ以上の2群間でのCOIの有意差の有無を検討した．

(4) 同様に，術前の体重身長比(Weight for Height：W/H) を1ずつ整数値で区切り，そのカットオフ値未満とそれ以上の2群間におけるCOIの有意差の有無を比較検討した．

(5) Waterlow分類[2,3]により，対象を4群に分け（図1），これら4群間でのCOIの有意差の有無を検討した．

2. 方法2　術前の病態の重症度がCOIの影響因子となり得るか否かの検討

(1) 対象をチアノーゼの有無によりチアノーゼ群と非チアノーゼ群の2群に分け，2群間でCOI，H/A，W/Hの有意差の有無を検討した．

(2) 術前の病態の重症度とCOIとの相関性の有無を検討した．ここで病態の重症度の指標にはPediatric Risk of Mortality score Ⅲ (PRISM Ⅲ)[4]を用いた．

検討方法としては，各対象毎に算出したPRISM Ⅲのスコアを1ずつ整数値で区切り，その2群間でのCOIの有意差の有無を検討した．

(3) 算出したPRISM Ⅲのスコアと，各対象毎のH/A，W/HおよびCOIとの相関性の有無を検討した．

3. 方法3　手術侵襲の大きさがCOIの影響因子となり得るか否かの検討

手術侵襲の大きさ（侵襲度）を厚美の分類[5]により，重症群と軽症群の2群に分け，2群間でのCOIの有意差の有無を検討した．

4. 方法4

さらにもし栄養状態，病態の重症度，手術侵襲の大きさ（侵襲度）によって分けた2群間でCOIに有意差がある場合，それら2群間の臨床的な背景に差がある可能性が危惧された．そこで方法1～3の検討で有意差の出た項目において2群間の背景を検討する目的で，このCOIの有意差に影響している可能性のある術前の身体プロフィール，PNI[1,6]を抽出し比較検討した．

統計学的処理

方法1 (1)～(4)，方法2 (1)，(2) および方法

表 2 H/A 別にみたアウトカム指標の比較（mean ± SD）

(1) ICU在室日数

カットオフ値	92	93	94	95	96	97	98	100	101	104	108
H/A高値群*	11.1±7.2 (n=20)	11.1±7.4 (n=19)	11.4±7.5 (n=18)	10.6±7.3 (n=16)	9.7±6.6 (n=15)	10.4±6.9 (n=13)	11.0±6.8 (n=12)	12.4±7.2 (n=9)	12.7±8.3 (n=7)	10.7±6.9 (n=6)	8.4±4.5 (n=5)
H/A低値群*	7.2±3.0 (n=5)	7.7±2.9 (n=6)	7.3±2.8 (n=7)	9.8±5.8 (n=9)	11.2±7.1 (n=10)	10.2±6.8 (n=12)	9.6±6.8 (n=13)	9.1±6.3 (n=16)	9.3±6.0 (n=18)	10.2±6.8 (n=19)	10.8±7.2 (n=20)
p	0.259	0.110	0.055	0.786	0.586	0.937	0.616	0.234	0.266	0.875	0.495

(2) 術後在院日数

カットオフ値	92	93	94	95	96	97	98	100	101	104	108
H/A高値群*	53.9±47.5 (n=20)	45.5±29.7 (n=19)	46.7±30.0 (n=18)	45.1±30.6 (n=16)	44.2±31.4 (n=15)	45.8±33.5 (n=13)	47.5±34.5 (n=12)	56.2±35.9 (n=9)	63.4±37.5 (n=7)	52.8±27.3 (n=6)	47.2±26.3 (n=5)
H/A低値群*	24.2±5.6 (n=5)	55.8±77.6 (n=6)	51.1±72.0 (n=7)	53.1±63.3 (n=9)	53.6±59.7 (n=10)	50.3±54.7 (n=12)	48.4±52.8 (n=13)	43.3±48.4 (n=16)	41.9±45.8 (n=18)	46.4±48.6 (n=19)	48.2±48.0 (n=20)
p	0.013	0.761	0.880	0.727	0.655	0.809	0.961	0.493	0.282	0.763	0.967

(3) 人工呼吸器管理期間（時間）

カットオフ値	92	93	94	95	96	97	98	100	101	104	108
H/A高値群*	96.7±105.5 (n=20)	97.2±108.4 (n=19)	101.3±110.0 (n=18)	98.5±109.3 (n=16)	79.0±79.6 (n=15)	85.8±83.5 (n=13)	92.4±83.6 (n=12)	104.3±94.2 (n=9)	98.1±106.5 (n=7)	68.7±79.5 (n=6)	70.6±88.7 (n=5)
H/A低値群*	47.2±48.1 (n=5)	53.8±46.0 (n=6)	49.6±43.5 (n=7)	66.1±75.3 (n=9)	98.5±124.6 (n=10)	87.9±115.6 (n=12)	81.6±112.9 (n=13)	76.9±101.8 (n=16)	82.4±97.4 (n=18)	92.5±104.5 (n=19)	90.9±102.0 (n=20)
p	0.323	0.356	0.106	0.439	0.638	0.959	0.790	0.514	0.727	0.614	0.689

*H/A高値群：H/Aが境界値以上
H/A低値群：H/Aが境界値未満

表 3 月齢別にみた H/A

月齢の境界値（カ月）	0	1	2	3	4	7	11
月齢低値群*	108.4±11.9 (n=9)	107.0±12.1 (n=10)	106.2±11.8 (n=11)	103.2±12.3 (n=14)	101.2±12.1 (n=17)	100.6±11.6 (n=19)	100.5±11.3 (n=20)
月齢高値群*	94.5±4.8 (n=16)	94.5±4.9 (n=15)	94.2±5.0 (n=14)	94.8±4.4 (n=11)	95.9±3.7 (n=8)	96.0±4.1 (n=6)	95.5±4.4 (n=5)
p	0.008	0.010	0.008	0.031	0.115	0.161	0.349

＊月齢低値群：境界値以下の月齢
月齢高値群：境界値より大きい月齢

3，4における2群間の統計学的処理手法には，対応のないt検定を用いた．また方法1（5）における4群間の統計学的処理手法にはANOVA(分散分析）を用いた．ここで，$p<0.05$を統計学的に有意差ありと判定した．数値はmean±SDで表記した．

結果

1. 結果1 術前の栄養状態がCOIの影響因子となり得るか否かの検討の結果

(1) 算出されたH/Aの最低値は86.1，最高値は125.4であった．そこでこの最低値と最高値を含む86～126の範囲で検討した．ここでカットオフ値91以下および109以上は，対象数が5例未満と少なく統計処理の行えないため，今回の検討からは除外した．

検討の結果，COIとしての術後在院日数にのみ，H/Aのカットオフ値を92に設定した場合に，統計学的有意差を認めた（表2）．

一方，ICU在室日数および人工呼吸器管理期間では，すべてのカットオフ値で有意差を認めなかった．

(2) H/Aのカットオフ値を0，1，2，3カ月とした場合にのみ，その月齢未満の群とその月齢以上の群の2群間でのH/Aに有意差を認めた（表3）．

(3) 栄養障害が認められ始める生後4カ月齢以上の対象に限定して，H/A別のアウトカム指標の比較検討を行った．

すなわちH/Aのカットオフ値を，3カ月以

表4　W/H別にみたアウトカム指標の比較（mean±SD）

(1) ICU在室日数

カットオフ値	79	80	81	82	84	87	89	92	100
H/A高値群*	10.3±7.0 (n=18)	10.6±7.1 (n=17)	10.3±6.7 (n=14)	9.8±6.7 (n=13)	9.6±7.0 (n=12)	9.8±7.5 (n=10)	10.0±7.9 (n=9)	10.6±8.2 (n=8)	9.6±7.3 (n=5)
H/A低値群*	9.2±7.5 (n=5)	8.7±6.8 (n=6)	9.8±7.7 (n=9)	10.4±7.5 (n=10)	10.6±7.2 (n=11)	10.3±6.8 (n=13)	10.1±6.5 (n=14)	9.8±6.4 (n=15)	10.2±7.0 (n=18)
p	0.754	0.571	0.868	0.854	0.725	0.866	0.963	0.793	0.864

(2) 術後在院日数

カットオフ値	79	80	81	82	84	87	89	92	100
H/A高値群*	39.9±28.8 (n=18)	40.3±29.6 (n=17)	40.1±32.2 (n=14)	37.5±31.9 (n=13)	38.9±32.8 (n=12)	42.0±35.4 (n=10)	44.6±36.5 (n=9)	47.3±38.1 (n=8)	38.4±25.3 (n=5)
H/A低値群*	45.6±29.7 (n=5)	43.5±27.0 (n=6)	42.7±23.0 (n=9)	45.9±23.9 (n=10)	43.5±24.0 (n=11)	40.5±23.2 (n=13)	38.9±23.0 (n=14)	37.9±22.6 (n=15)	41.9±29.9 (n=18)
p	0.701	0.818	0.841	0.493	0.706	0.901	0.654	0.463	0.814

(3) 人工呼吸器管理期間（時間）

カットオフ値	79	80	81	82	84	87	89	92	100
H/A高値群*	96.4±104.3 (n=18)	99.3±106.8 (n=17)	88.8±81.3 (n=14)	78.0±73.5 (n=13)	71.5±72.8 (n=12)	67.8±77.6 (n=10)	72.9±80.5 (n=9)	79.0±83.8 (n=8)	54.0±33.1 (n=5)
H/A低値群*	67.2±95.4 (n=5)	64.0±85.7 (n=6)	92.1±131.6 (n=9)	105.8±131.4 (n=10)	110.4±125.6 (n=11)	107.2±116.1 (n=13)	101.1±113.8 (n=14)	96.0±111.5 (n=15)	100.1±111.7 (n=18)
p	0.579	0.475	0.941	0.526	0.369	0.366	0.526	0.710	0.142

＊W/H高値群：W/Hが境界値以上
W/H低値群：W/Hが境界値未満

表5　N群，S群，W群，SW群の4群間のアウトカム指標の比較（mean±SD）

アウトカム指標	N群 (n=14)	S群 (n=3)	W群 (n=4)	SW群 (n=2)
ICU在室日数（日）	11.1±7.7	8.3±3.5	10.3±8.2	5.5±0.7
術後在院日数（日）	44.3±31.3	21.7±3.8	51.3±31.0	28.0±7.1
人工呼吸器管理期間（時間）	108.8±113.3	55.0±65.1	78.3±106.4	35.5±17.7

有意差なし

下の症例を含めた25例で有意差の認められたH/A=92とした場合，月齢3カ月以下の症例を除いた11例においては術後在院日数に有意差を認めなかった（術後在院日数：H/A低値群 vs H/A高値群=26.0±7.0 vs 32.3±13.3，p=0.470）．すなわちH/Aは，今回対象とした心臓手術を施行された乳幼児のCOIとしては利用できないことが明らかとなった．

(4) 算出されたW/Hの最低値は70.1，最高値は120.4であった．そこでこれら最低値と最高値を検定数値範囲に含むように70～121を検定範囲に設定し，この範囲を整数値で1ずつ区切り，その整数値をカットオフ値とした．次にこのカットオフ値のその値未満とその値以上の2群に分け，2群間で3種類のCOIを比較検討した．ただしここでカットオフ値77以下および104以上はそれぞれ対象数が5例未満と少なく統計処理が行えないため，今回の検討から除外した．

その結果，COIの3指標すべてにおいて2群間で有意差を認めなかった（表4）．

(5) N群，S群，W群，SW群の4群間でCOIの比較検討を行った結果，4群間でCOIには統計学的有意差は認めなかった（表5）．

2．結果2　術前の病態の重症度がCOIの影響因子となり得るか否かの検討の結果

(1) チアノーゼの有無により分けた2群間で，COI，H/AおよびW/Hを比較検討した結果，

表6 チアノーゼの有無から見たアウトカム指標の比較（mean±SD）

	チアノーゼ群 n=14	非チアノーゼ群 n=11	p
ICU在室日数（日）	9.0±5.2	11.6±8.2	0.291
在院日数（日）	45.1±51.4	51.6±34.5	0.720
人工呼吸器管理期間（時間）	56.1±45.7	125.9±131.7	0.119
H/A(%)	98.1±9.9	101.3±11.2	0.462
W/H(%)	94.2±12.3	81.2±9.1	0.009

表7 PRISM Ⅲスコア別に見たアウトカム指標の比較（mean±SD）

アウトカム指標	PRISMⅢ ≦0 (n=7)	PRISMⅢ >0 (n=13)	p
ICU在室日数(日)	6.9±2.0	12.6±8.1	0.028
術後在院日数（日）	32.9±20.1	61.4±56.6	0.116
人工呼吸器管理時間（時間）	43.3±29.7	109.0±120.1	0.082

アウトカム指標	PRISMⅢ ≦1 (n=8)	PRISMⅢ >1 (n=12)	p
ICU在室日数(日)	7.6±2.8	12.6±8.4	0.080
術後在院日数（日）	31.3±19.1	64.8±56.6	0.078
人工呼吸器管理時間（時間）	57.4±48.4	105.0±124.6	0.250

アウトカム指標	PRISMⅢ ≦3 (n=14)	PRISMⅢ >3 (n=6)	p
ICU在室日数(日)	8.9±5.4	14.5±9.5	0.110
術後在院日数（日）	46.2±51.0	63.5±40.3	0.472
人工呼吸器管理時間（時間）	72.4±100.2	117.6±108.6	0.379

表8 PRISM Ⅲとの相関係数

	H/A	W/H	ICU在室日数	術後在院日数	人工呼吸器の管理期間
r=	0.022	-0.028	0.447	0.330	0.289
p=	n.s.	n.s.	0.048	n.s.	n.s.

表9 手術侵襲の大きさ別にみたアウトカム指標の比較（mean±SD）

アウトカム指標	手術の大きさ 重症群 (n=16)	軽症群 (n=9)	p
ICU在室日数（日）	10.8±7.0	9.4±6.4	0.650
術後在院日数（日）	60.6±50.5	25.6±12.4	0.017
人工呼吸器管理期間（時間）	79.5±86.8	99.7±120.0	0.665

表10 H/A＝92未満群，以上群の身体プロフィール（mean±SD）

H/A	<92	92≦	p
n（男児/女児）	5 (5/0)	20 (13/7)	
月齢（カ月）	6.4±6.5	5.0±6.5	0.659
身長（cm）	59.5±6.7	61.4±10.7	0.711
体重（kg）	5.0±1.4	5.4±2.3	0.689
身長年齢比（H/A，%）	88.3±1.5	102.3±9.8	<0.001
体重身長比（W/H，%）	86.7±12.7	88.4±12.8	0.792
PRISMⅢ	3.0±4.2	3.4±3.5	0.857
手術の大きさ 重症/総数(%)	2/6 (33.3)	14/19 (73.7)	0.079
PNI	55.8±7.4	60.3±15.6	0.539

W/Hに有意差を認めた（表6）．

その他の指標には有意差を認めなかった．

(2) 術前のPRISM Ⅲスコアを1ずつの整数値で区切り，その整数値未満とそれ以上の2群に対象を分けた．この2群間でCOIを比較検討した．なお対象数が5例未満となるカットオフ値は統計学的処理が行えないため，今回の検討からは除外した．

検討の結果，術前のPRISM Ⅲスコアが0点の群と1点以上の群の2群間でのみICU在室日数に有意差がでた（表7）．すなわちPRISM Ⅲスコア1点以上の群で有意にICU在室日数が長かった．

(3) PRISM Ⅲのスコアと，術前のH/A，W/HおよびCOIの3つの指標の相関係数を表8に示す．

その結果，PRISM Ⅲスコアと ICU在室日数との間に相関関係が認められた．それ以外の項目であるH/A，W/Hおよび術後在院日数，人工呼吸器管理期間との間には相関関係を認めなかった．

3. 結果3 手術侵襲の大きさがCOIの影響因子となり得るか否かの検討の結果

厚美の分類により，術式を重症群と軽症群の2群に分け，COIの比較検討を行った．その結果，手術侵襲の大きさとCOIとしての術後在院日数との間に統計学的有意差を認めた（表9，$p=0.017$）．一方，手術侵襲の大きさとICU在室日数および人工呼吸器管理期間との間に有意差を認めなかった．

4. 結果4

(1) 方法1～3の検討で術後在院日数に有意差の出たH/Aカットオフ値92での2群間に対して，身体プロフィール，PNIを検討した．その結果，H/A低値群と高値群の2群間に有意差を認めなかった（表10）．

表11　手術侵襲の大きさ別身体プロフィール（mean ±SD）

厚美の分類[5]	重症群	軽症群	p
n（男児/女児）	16（11/5）	9（7/2）	−
月齢（カ月）	4.9±7.3	5.9±4.7	0.711
身長（cm）	59.8±10.9	63.2±7.9	0.418
体重（kg）	5.2±2.4	5.6±1.6	0.705
身長年齢比（H/A，%）	102.2±11.7	94.8±5.4	0.042
体重身長比（W/H，%）	90.9±13.4	83.6±10.1	0.179
PRISMⅢ	3.9±4.0	2.0±2.0	0.299
PNI	57.9±14.1	62.2±15.1	0.487

(2) 同様に有意差の出た厚美の分類による重症群，軽症群の2群間での身体プロフィール，PNIの比較検討を行った．

その結果H/Aに有意差を認めたが，それ以外の項目においては有意差を認めなかった（表11）．

考察

術前に栄養障害がある患者では，創傷治癒の遅延や術後感染症発生の危険性が増すなど術後アウトカムの不良化がみられ，特に外科領域で予後判定栄養アセスメントとして，予後推定栄養指数が報告されている[6)~10)]．

一般に栄養アセスメントを行う目的は，有害事象を高率に発症する確率（リスク）の高いハイリスク群を判別し，予後あるいは治療効果を推定することといえる．すなわち予後判定ツールの意義のひとつに栄養アセスメントがある．したがって術前に患児の栄養状態のアセスメントを行うことは，栄養障害がある症例を判別し，これらハイリスク症例に対し，より濃密な栄養サポートを施行し，術後アウトカムを改善させるために重要である．

従来より，小児の栄養障害の指標として報告されたWaterlow分類は，身体計測値である年齢，身長，体重をもとに分類している[2),3)]．Waterlowはその原著において，(a) normal（図1のN群），(b) malnourished but not retarded（図1のW群），(c) malnourished and retarded（図1のSW群），(d) retarded but not malnourished（図1のS群）の4つのカテゴリーに分けている[2)]．これらのうち，一般的にカテゴリー（b）群は急性の栄養障害を示し，またカテゴリー（c）群は慢性の栄養障害を示すとされてきた．

今回我々は，小児の栄養障害の指標であるH/A，W/HおよびWaterlowの分類が術後のアウトカム指標となり得るか検討した．

その結果，心臓手術乳幼児（月齢0-18カ月）を対象として，W/H単独では，いずれのカットオフ値においても今回選択したアウトカム指標，すなわちICU在室日数，術後在院日数，人工呼吸器管理期間に有意差を認めなかった．したがって，従来より栄養状態の判定指標として小児において頻用されてきたこの栄養指標は，今回の対象群においてはアウトカム予測指標とはなり得ないといえる．

一方，H/Aのカットオフ値で分けた2群間で，アウトカム指標としての術後在院日数に有意差を認めた．しかしここで例えば胎児期に重篤な栄養障害がない限り，出生直後から短期間では栄養障害指標としてのH/Aには現れず，したがって出生後早期の乳児期では栄養状態をH/Aが正しく反映しない可能性が危惧された．

方法1（2）の結果は，今回の対象においては栄養不良がH/A値に現れるには，3カ月を要することを意味していると考えられた．いいかえると生後3カ月以下の小児においては，従来用いられているH/Aによっては出生後早期の乳児栄養不良を抽出できず，したがって乳児の栄養障害を見落とす危険性のあることが示されたものと考えられる．

チアノーゼの有無により分けた2群間では，チアノーゼ群は非チアノーゼ群と比べ，有意にW/Hが高かった．これは検討前，チアノーゼが栄養障害発生に影響するとの我々の予想に反するものであった．すなわち少なくとも今回の対象群においては，チアノーゼの存在が栄養状態に影響を与えていないと考えられた．

今回の検討では，ICU在室日数とPRISMⅢス

表12 PRISM Ⅲ各項目の合計スコアに対する割合

項目	合計スコア	%
PaO_2	24	36.4
BUN	15	22.7
アシドーシス	8	12.1
$PaCO_2$	5	7.6
Plt	4	6.1
収縮期血圧	3	4.5
PT (PTT)	3	4.5
pH	2	3.0
クレアチニン	2	3.0
合計	66	100

表13 本研究の結果のまとめ：H/A，W/Hおよびチアノーゼ，PRISM Ⅲ，手術侵襲の大きさとアウトカム予測の有意差の有無

	H/A	W/H	チアノーゼ	PRISMⅢ	手術侵襲の大きさ（厚美の分類）
ICU在室日数（日）	×	×	×	○	×
術後在院日数（日）	×	×	×	×	○
人工呼吸器管理期間（時間）	×	×	×	×	×

○：有意差あり（$p<0.05$），×：有意差なし

コアとの間に相関が認められた．PRISM Ⅲを整数値をカットオフ値に設定し，そのカットオフ値以下とそれより上の2群を比較した．その結果カットオフ値を0に設定したときのみ，スコアが1点以上の群でICU在室日数が有意に長かった．

ここでスコア1以上の群（n＝13）で，PRISM Ⅲの一体どの項目がスコアの点数の加点に関与したか検討する目的で，スコア1点以上の症例の全スコアを検討した．スコア1点以上の全症例13例のPRISM Ⅲの17項目の各スコアを，項目毎に抽出し，各項目毎に合計したところ，PaO_2が全スコアの36.4％，BUNが全スコアの22.7％，アシドーシスが全スコアの12.1％であった（表12）．すなわちこれら3項目のスコアで全体のスコアの71％を占めていた．いいかえると17項目あるPRISM Ⅲのうちのこの3項目が，今回の心疾患の重症度を重症化していることが示唆された．今回の対象においてはPRISM Ⅲスコアを高得点にするこの3項目は，心肺機能障害，腎機能障害，末梢循環不全であり，いずれも心不全徴候と関連する指標であり，今回の対象特異性が高いのかもしれない．

しかし対象数に限りがあるため，確定的な結論を出すには，今後のさらなる症例の集積による検討が必要であろう．

一方，厚美の分類による[5]手術侵襲の大きさ（侵襲度）は，術後の在院日数を予測する指標となる可能性が示された．

以上，本研究の結果をまとめた（表13）．

また月齢3カ月以下の心疾患を有する乳児に対しては，従来の栄養指標を用いて栄養障害を今後発生するリスクのある症例（本稿においては，仮に“アットリスク症例”と呼ぶ）[1]を予測することはできない．したがって適切な小児用栄養アセスメント指標の開発が急務と考えられた．

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Waterlow's Nutritional Indices are not Outcome Indicators in Infantile Patients Undergoing Cardiac Surgery

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The aim of our study was to examine whether clinical outcome indicators, which include the length of stay in the ICU, postoperative hospital days (POH), and the respiratory management duration, were compared with the Warerlow classification, PRISM Ⅲ and the stress intensity of the operation. When setting a cut off value of H/A at 92, POH was statistically longer in the high H/A group than in low the H/A group. However, there is the possibility that H/A might not reflect nutritional status in small infants. So, H/A was examined by dividing the patients into two groups : a younger and older group divided by months of age. The result was that H/A did not differ in infants older than 3 months. However, POH was significant different when the two were divided into subsets : mild and severe, according to the stress intensity of the operation. From our results, it was concluded that nutritional indices (H/A, W/H) should not be utilized as outcome indicators in infants undergoing cardiac surgery.

Infants, Waterlow classification, Outcome Index, PRISM Ⅲ, At risk

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An *N*-Glycosylation Site on the β-Propeller Domain of the Integrin α5 Subunit Plays Key Roles in Both Its Function and Site-specific Modification by β1,4-*N*-Acetylglucosaminyltransferase III*

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Recently we reported that *N*-glycans on the β-propeller domain of the integrin α5 subunit (S-3,4,5) are essential for α5β1 heterodimerization, expression, and cell adhesion. Herein to further investigate which *N*-glycosylation site is the most important for the biological function and regulation, we characterized the S-3,4,5 mutants in detail. We found that site-4 is a key site that can be specifically modified by *N*-acetylglucosaminyltransferase III (GnT-III). The introduction of bisecting GlcNAc into the S-3,4,5 mutant catalyzed by GnT-III decreased cell adhesion and migration on fibronectin, whereas overexpression of *N*-acetylglucosaminyltransferase V (GnT-V) promoted cell migration. The phenomenon is similar to previous observations that the functions of the wild-type α5 subunit were positively and negatively regulated by GnT-V and GnT-III, respectively, suggesting that the α5 subunit could be duplicated by the S-3,4,5 mutant. Interestingly GnT-III specifically modified the S-4,5 mutant but not the S-3,5 mutant. This result was confirmed by erythroagglutinating phytohemagglutinin lectin blot analysis. The reduction in cell adhesion was consistently observed in the S-4,5 mutant but not in the S-3,5 mutant cells. Furthermore mutation of site-4 alone resulted in a substantial decrease in erythroagglutinating phytohemagglutinin lectin staining and suppression of cell spread induced by GnT-III compared with that of either the site-3 single mutant or wild-type α5. These results, taken together, strongly suggest that *N*-glycosylation of site-4 on the α5 subunit is the most important site for its biological functions. To our knowledge, this is the first demonstration that site-specific modification of *N*-glycans by a glycosyltransferase results in functional regulation.

Glycosylation is a crucial post-translational modification of most secreted and cell surface proteins (1). Glycosylation is involved in a variety of physiological and pathological events, including cell growth, migration, differentiation, and tumor invasion. It is well known that glycans play important roles in cell-cell communication, intracellular signal transduction, protein folding, and stability (2, 3).

Integrins comprise a family of receptors that are important for cell adhesion. The major function of integrins is to connect cells to the extracellular matrix, activate intracellular signaling pathways, and regulate cytoskeletal formation (4). Integrin α5β1 is well known as a fibronectin (FN)[3] receptor. The interaction between integrin α5 and FN is essential for cell migration, cell survival, and development (5–8). In addition, integrins are *N*-glycan carrier proteins. For example, α5β1 integrin contains 14 and 12 putative *N*-glycosylation sites on the α5 and β1 subunits, respectively. Several studies suggest that *N*-glycosylation is essential for functional integrin α5β1. When human fibroblasts were cultured in the presence of 1-deoxymannojirimycin, which prevents *N*-linked oligosaccharide processing, immature α5β1 integrin appeared on the cell surface, and FN-dependent adhesion was greatly reduced (9). Treatment of purified integrin α5β1 with *N*-glycosidase F, which cleaves between the innermost *N*-acetylglucosamine (GlcNAc) and asparagine *N*-glycan residues of *N*-linked glycoproteins, prevented the inherent association between subunits and blocked α5β1 binding to FN (10).

A growing body of evidence indicates that the presence of the appropriate oligosaccharide can modulate integrin activation. *N*-Acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of GlcNAc to mannose that is β1,4-linked to an underlying *N*-acetylglucosamine, producing what is known as a "bisecting" GlcNAc linkage as shown in Fig. 1*B*. GnT-III is generally regarded as a key glycosyltransferase in *N*-glycan biosynthetic pathways and contributes to inhibition of metastasis. The introduction of a bisecting GlcNAc catalyzed by GnT-III suppresses additional processing and elongation of *N*-glycans.

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[3] The abbreviations used are: FN, fibronectin; BSA, bovine serum albumin; E4-PHA, erythroagglutinating phytohemagglutinin; GFP, green fluorescent protein; GlcNAc, *N*-acetylglucosamine; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; L4-PHA, leukoagglutinating phytohemagglutinin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; RT-CES, real time cell electronic sensing; WT, wild type; POMT, protein *O*-mannosyltransferase.

These reactions, which are catalyzed *in vitro* by other glycosyltransferases, such as *N*-acetylglucosaminyltransferase V (GnT-V), which catalyzes the formation of β1,6 GlcNAc branching structures (Fig. 1*B*) and plays important roles in tumor metastasis, do not proceed because the enzymes cannot utilize the bisected *N*-glycans as a substrate. Introduction of the bisecting GlcNAc to integrin α5 by overexpression of GnT-III resulted in decreased in ligand binding and down-regulation of cell adhesion and migration (11–13). Contrary to the functions of GnT-III, overexpression of GnT-V promoted integrin α5β1-mediated cell migration on FN (14). These observations clearly demonstrate that the alteration of *N*-glycan structure affected the biological functions of integrin α5β1. Similarly characterization of the carbohydrate moieties in integrin α3β1 from non-metastatic and metastatic human melanoma cell lines showed that expression of β1,6 GlcNAc branched structures was higher in metastatic cells compared with non-metastatic cells, confirming the notion that the β1,6 GlcNAc branched structure confers invasive and metastatic properties to cancer cells. In fact, Partridge *et al.* (15) reported that GnT-V-modified *N*-glycans containing poly-*N*-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis, promoting intracellular signaling and consequently cell migration and tumor metastasis.

In addition, sialylation on the non-reducing terminus of *N*-glycans of α5β1 integrin plays an important role in cell adhesion. Colon adenocarcinomas express elevated levels of α2,6 sialylation and increased activity of ST6GalI sialyltransferase. Elevated ST6GalI positively correlated with metastasis and poor survival. Therefore, ST6GalI-mediated hypersialylation likely plays a role in colorectal tumor invasion (16, 17). In fact, oncogenic *ras* up-regulated ST6GalI and, in turn, increased sialylation of β1 integrin adhesion receptors in colon epithelial cells (18). However, this is not always the case. The expression of hyposialylated integrin α5β1 was induced by phorbol ester-stimulated differentiation in myeloid cells in which the expression of the ST6GalI was down-regulated by the treatment, increasing FN binding (19). A similar phenomenon was also observed in hematopoietic or other epithelial cells. In these cells, the increased sialylation of the β1 integrin subunit was correlated with reduced adhesiveness and metastatic potential (20–22). In contrast, the enzymatic removal of α2,8-linked oligosialic acids from the α5 integrin subunit inhibited cell adhesion to FN (23). Collectively these findings suggest that the interaction of integrin α5β1 with FN is dependent on its *N*-glycosylation and the processing status of *N*-glycans.

Because integrin α5β1 contains multipotential *N*-glycosylation sites, it is important to determine the sites that are crucial for its biological function and regulation. Recently we found that *N*-glycans on the β-propeller domain (sites 3, 4, and 5) of the integrin α5 subunit are essential for α5β1 heterodimerization, cell surface expression, and biological function (24). In this study, to further investigate the underlying molecular mechanism of GnT-III-regulated biological functions, we characterized the *N*-glycans on the α5 subunit in detail using genetic and biochemical approaches and found that site-4 is a key site that can be specifically modified by GnT-III.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—A monoclonal antibody against human integrin α5 subunit (clone1) for Western blot analysis was obtained from BD Biosciences. For immunoprecipitation, the agarose-conjugated anti-green fluorescent protein (GFP) antibody (RQ2) was obtained from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Peroxidase-conjugated anti-mouse IgG was obtained from Cell Signaling Technology, Inc. (Danvers, MA). A VECTASTAIN ABC kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). Antibodies against GnT-III (33A8) and GnT-V (24B11) were obtained from FUJIREBIO Inc. (Tokyo, Japan). Biotinylated erythroagglutinating phytohemagglutinin (E4-PHA), biotinylated leukoagglutinating phytohemagglutinin (L4-PHA), and biotinylated *Datura stramonium* lectin were purchased from Seikagaku Corp. (Tokyo, Japan). For fluorescence-activated cell sorting analysis, mouse anti-human α5β1 integrin monoclonal antibody (HA5, MAB1999) was purchased from Chemicon (Temecula, CA).

Cells and Cell Culture—The integrin α5 subunit-deficient CHO K1 cell line (CHO-B2) was a gift from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill, NC) (25). The CHO-B2 stable expression cells containing various integrin α5 with altered *N*-glycosylation sites were established in our laboratory (24). As shown in Fig. 1*A*, wild type (WT) indicates CHO-B2 expressing wild-type (full *N*-glycosylation sites) integrin α5; S-3,4,5, S-3,5, and S-4,5 show that all *N*-glycosylation sites were removed with site-directed mutagenesis except the indicated sites; and D-3 or D-4 represent single mutations at the indicated site. A HeLa cell line was purchased from RIKEN BioResource Center (Tsukuba, Japan). The stable expression of S-3,5 and S-4,5 mutants in HeLa cells was obtained by viral expression vector as mentioned below. These mutants and cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, non-essential amino acids (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Nacalai Tesque, Inc., Kyoto, Japan) under a humidified atmosphere containing 5% CO_2.

GnT-III, GnT-V, and α5 Mutant (S-3,5 and S-4,5) Expression with Viral Vectors—The cDNAs encoding human GnT-III and GnT-V were amplified for cloning into pENTR-D-Topo for the Gateway Conversion System (Invitrogen) according to the manufacturer's protocol. The cloned genes were inserted into the virus expression vector, pBABE-puro (Addgene, Inc. Cambridge, MA), accommodated into the Gateway Conversion System using LR Clonase reaction. The GnT-III and GnT-V constructs were transfected into Phoenix-Ampho cells with Lipofectamine 2000 (Invitrogen) for production of viral supernatants. The various α5 integrin mutants were infected with the resulting viral supernatant containing 10 μg/ml Polybrene (Sigma-Aldrich) and selected with 13 μg/ml puromycin for 2 weeks. In the case of HeLa cells expressing S-3,5 and S-4,5 mutants, after virus infection the infected cells were selected with 2.5 μg/ml puromycin. For mock transfection, the same protocol was performed using the empty virus expression vector only.

Cell Adhesion Assay Using 96-well Plate—96-well plates (Corning Inc.) were coated with 3 μg/ml FN at 37 °C for 1 h and blocked with 1% bovine serum albumin (BSA) in DMEM at 37 °C for 1 h. The cells were detached with trypsin containing 1 mM EDTA, resuspended with 0.5 mg/ml trypsin inhibitor (Nacalai Tesque, Inc.) in DMEM. The suspended cells were centrifuged at 1,000 rpm for 3 min and diluted to 4×10^5 or 8×10^5 cells/ml with assay medium, 0.1% BSA in DMEM. One hundred-microliter aliquots of cell suspension were added to each well, and the plates were incubated at 37 °C for 20–25 min. After incubation, attached cells were fixed with 25% glutaraldehyde (Nacalai Tesque, Inc.) and stained with 0.5% crystal violet. The absorbance at 590 nm was measured using an automated microtiter plate spectrometer, Powerscan® HT (Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) operated with Microplate Data Analysis Software, KC4™ (BioTek Instruments, Inc., Winooski, VT). Cell spreading assays were performed as described previously (12, 24). After a 20-min incubation, representative fields were observed using phase-contrast microscopy, and spread cells were counted. The rounded cells were not considered as spread cells.

Cell Adhesion Kinetics Assay Using the Real Time Cell Electronic Sensing (RT-CES™) System—The cell adhesion kinetics assay was performed using a RT-CES system (ACEA Biosciences, Inc.) (26). Briefly ACEA Biosciences, Inc. electrosensing 16-well plates were coated with 50 μl of 10 μg/ml FN (Sigma) at 37 °C for 1 h and then blocked with 1% BSA in DMEM at 37 °C for 1 h. The cells were detached with trypsin containing 1 mM EDTA and resuspended with 0.5 mg/ml trypsin inhibitor (Nacalai Tesque, Inc.) in DMEM. The suspended cells were centrifuged at 1,000 × *g* for 3 min and diluted to 1×10^5 or 8×10^5 cells/ml with assay medium (0.1% BSA in DMEM). Fifty-microliter aliquots of the cell suspension were added to each well, and the assay was performed using RT-CES SP software. The program was set up such that the cell index was measured every 2 min for 3 h.

Cell Migration—Transwells (BD BioCoat™ Control Inserts, 8.0-μm inserts; BD Biosciences) were coated only on the bottom side with 10 μg/ml FN at 37 °C for 1 h. Cells were starved in serum-free medium for 4 h, trypsinized, and suspended with 0.5 mg/ml trypsin inhibitor (Nacalai Tesque, Inc.) in DMEM. Suspended cells were centrifuged, and supernatants were removed. The cell pellets were resuspended with assay medium (0.1% BSA in DMEM containing 1% fetal bovine serum) and diluted to 4×10^5 cells/ml. One hundred-microliter aliquots of the cell suspension were added to each FN-coated Transwell; the cells were then incubated at 37 °C for 3 h. After incubation, cells on the upper side were removed by scraping with a cotton swab. The membranes in the Transwells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 30 min. Cells that had migrated to the lower side were counted using a phase-contrast microscope.

Immunoprecipitation, Western Blot, and Lectin Blot—Subconfluent cells were washed with phosphate-buffered saline twice and lysed with ice-cold lysis buffer (1% Triton in Tris-buffered saline containing protease inhibitor mixture (Nacalai Tesque, Inc.)). The cell lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatants were obtained, and protein concentrations were determined using a BCA™ Protein Assay kit (Pierce). Equal amounts of protein were incubated with 10 μl of agarose-conjugated anti-GFP antibody and 15 μl of Sepharose™ 4B at 4 °C for 1 h. The immunocomplexes were washed twice with ice-cold lysis buffer, then were eluted with SDS sample buffer, and boiled for 5 min. The immunoprecipitates were subjected to 6.0 or 7.5% SDS-PAGE and then were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk or 5% BSA in Tris-buffered saline for Western blot and lectin blot, respectively. After blocking, the membranes were incubated with either primary antibody or lectin for 1 h. For Western blot, membranes were incubated with the secondary antibody conjugated with peroxidase for 1 h, and the immunoreactive proteins were visualized using an ECL kit (GE Healthcare). For lectin blot, the lectin-binding proteins were detected using a VECTASTAIN ABC kit and an ECL kit.

Flow Cytometry—Flow cytometry was performed as described previously (24). Briefly cells were detached by trypsinizing and incubated with mouse anti-human α5β1 integrin monoclonal antibody (HA5) followed by Alexa Fluor 647 anti-mouse IgG. Negative controls underwent the same procedure without primary antibody. The analyses were performed using a FACSCalibur instrument (BD Biosciences) equipped with CELLQuestPro software.

RESULTS

Comparison of N-Glycosylation Patterns on S-3,4,5 α5 Subunit Mutant in GnT-III and GnT-V Transfectants—*N*-Glycosylation is essential for integrin α5β1 heterodimer formation and therefore plays an important role in the biological function of integrin. GnT-III-modified integrin α5β1 decreased cell adhesion and cell migration on FN (12). In contrast to GnT-III, GnT-V specifically modified only the β1 subunit and up-regulated integrin α5β1-mediated cell migration (14). Recently we found that three *N*-glycosylation sites, sites 3, 4, and 5 from the N terminus of the α5 subunit, were essential for the biological functions of integrin, such as cell adhesion and migration on FN and heterodimerization.

The purpose of the present study was to determine whether the S-3,4,5 mutant, which contained only three potential *N*-glycosylation sites (*i.e.* sites 3, 4, and 5), had characteristics similar to those of the wild-type α5 subunit, such as modification by GnT-III and GnT-V as described above. Various α5 subunit mutants were used in this study as shown in Fig. 1*A*. First the expression levels of GnT-III and GnT-V in S-3,4,5 mutant cells that had been transfected with a retrovirus system were examined by Western blotting (Fig. 2*A*). Their products were detected by E4-PHA lectin, which specifically recognizes bisecting GlcNAc, and by L4-PHA lectin, which selectively recognizes β1,6-branching GlcNAc, blots (Fig. 2*B*) (27, 28). As expected, bands corresponding to GnT-III and GnT-V as well as lectin reactivities of E4-PHA and L4-PHA were increased in the GnT-III and GnT-V transfectants, respectively (Fig. 2, *A* and *B*). Equal amounts of protein (20 μg) were loaded in each lane, and α-tubulin was used as the loading control. Next we immunoprecipitated α5 and detected *N*-glycans using E4-PHA and L4-PHA lectin blotting (Fig. 2*C*). The E4-PHA reactivities

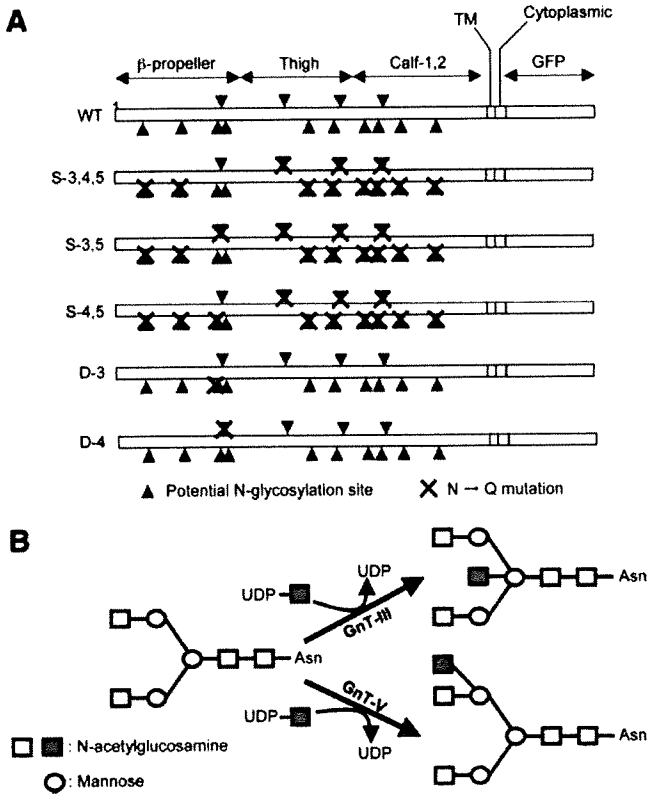

FIGURE 1. **Potential *N*-glycosylation sites on the α5 subunit and its modification by GnT-III and GnT-V.** *A*, schematic diagram of potential *N*-glycosylation sites on the α5 subunit. Putative *N*-glycosylation sites are indicated by *triangles*, and point mutations are indicated by *crosses* (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q). *B*, illustration of the reaction catalyzed by GnT-III and GnT-V. *Square*, GlcNAc; *circle*, mannose. *TM*, transmembrane domain.

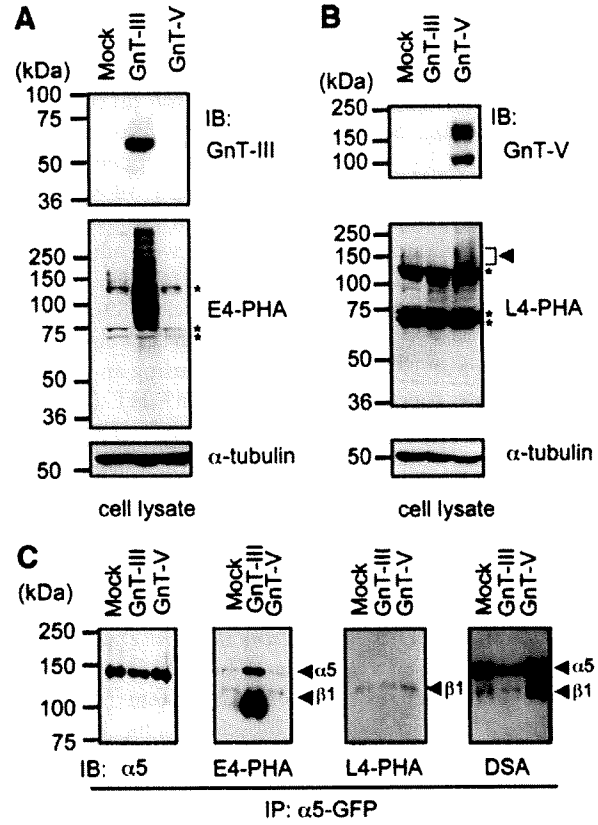

FIGURE 2. **Comparison of *N*-glycosylation patterns on α5 subunits modified by GnT-III and GnT-V.** GnT-III and GnT-V expression vectors were transfected into S-3,4,5 cells using the Phoenix retrovirus system, and stable expression cells were established as described under "Experimental Procedures." Confluent cells were harvested and lysed for immunoblotting (*IB*). Equal amounts of protein (20 μg) were separated by 7.5% SDS-PAGE under reducing conditions, and the membranes were probed with antibodies against GnT-III (*A*, *upper panel*) and GnT-V (*B*, *upper panel*) or with the E4-PHA (*A*, *middle panels*) and L4-PHA lectins (*B*, *middle panels*) and reprobed with anti-α-tubulin, which was used as loading control (*A* and *B*, *lower panels*). *Asterisks* indicate nonspecific staining for E4-PHA or L4-PHA. *C*, cell lysates were subjected to immunoprecipitation (*IP*) using agarose-conjugated anti-GFP antibody. The immunoprecipitates were subjected to 6.0% SDS-PAGE under non-reducing conditions, blotted, and probed with E4-PHA, L4-PHA, and *D. stramonium* (*DSA*) lectins or probed with antibody against the α5 subunit.

were much stronger in GnT-III transfectants than those in mock or GnT-V transfectants. This result indicates that both the α5 and β1 subunits are targets of GnT-III. In contrast, results of L4-PHA lectin staining indicated that only the β1 subunit could be modified by GnT-V, consistent with a previous study (14). The reactivities of *D. stramonium* lectin, which preferentially reacts with branched sugar chains (more than triantennary) (29), consistently showed a significant increase in the β1 subunit of GnT-V transfectants. A significant decrease in the α5 subunit of the GnT-III transfectants further supports the notion that introduction of GnT-III suppresses additional processing and branching formation of *N*-glycans catalyzed by other endogenous glycosyltransferases, such as GnT-V and GnT-IV. Although it is not clear whether the modification levels of branched *N*-glycans on α5 subunits were enhanced in GnT-V transfectants compared with the levels in mock transfectants, it could be argued that intrinsic GnT-V-mediated glycosylation is enough for occupation of some *N*-glycosylation sites on the α5 subunit, which may be specifically and exclusively modified by GnT-V.

Effects of GnT-III and GnT-V on Integrin-mediated Cell Adhesion and Migration in S-3,4,5 Transfectants—It is well known that wild-type integrin α5β1-mediated cell migration can be positively and negatively regulated by GnT-V and GnT-III, respectively. Therefore, we determined whether modifications of S-3,4,5 mutants could mimic wild-type α5 to affect its biological functions, such as cell adhesion and cell migration. As shown in Fig. 3*A*, cell adhesion on FN was down-regulated in GnT-III transfectants compared with mock and GnT-V transfectants. The cell adhesion kinetics assay using RT-CES also showed the same tendency (Fig. 3*B*). On the other hand, cell migration was determined using the Transwell assay as described under "Experimental Procedures." Interestingly overexpression of GnT-III significantly inhibited cell migration on FN, whereas GnT-V promoted cell migration relative to the mock transfectants (Fig. 3*C*). These results, taken together, suggest that the α5 subunit could be duplicated by the S-3,4,5 mutant. The up-regulation of cell migration in GnT-V transfectants could be ascribed to the *N*-glycans of the β1 subunit modified by GnT-V.

To determine whether overexpression of GnT-III or GnT-V affected integrin α5β1 expression on the cell surface, we per-

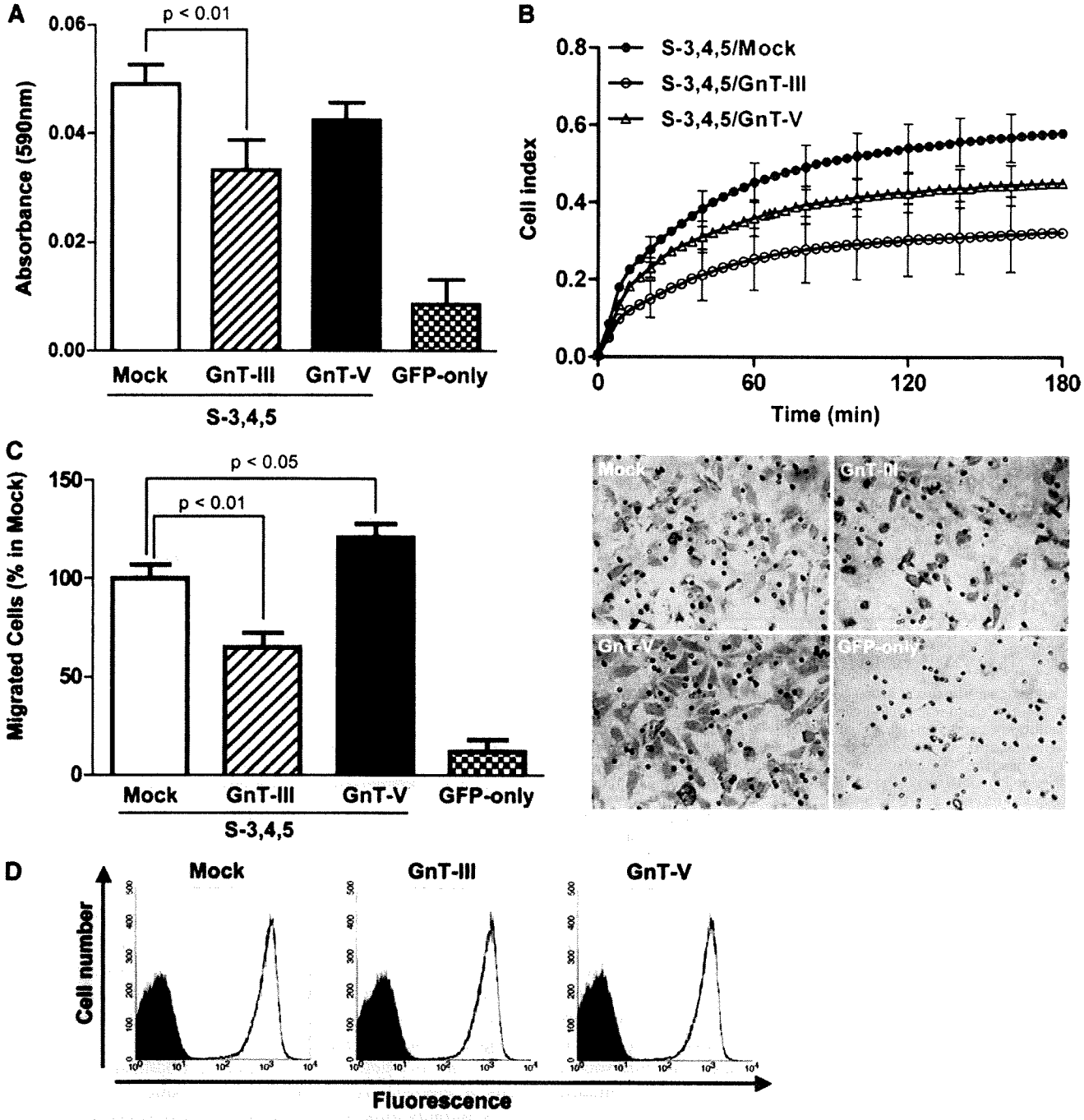

FIGURE 3. **Effects of overexpression of GnT-III and GnT-V on FN-mediated cell adhesion and migration in S-3,4,5 mutants.** *A*, subconfluent cells were detached, and 40,000 cells were added to the 96-well plates coated with 3 μg/ml FN for the cell adhesion assay. The plates were incubated at 37 °C for 20 min and then washed twice with warmed phosphate-buffered saline to remove non-adherent cells. The adherent cells were fixed with 25% glutaraldehyde and stained with 0.5% crystal violet, and then the absorbance at 590 nm was measured. The *bars* represent the S.D. *B*, cell adhesion kinetics assay using the RT-CES system. Subconfluent cells were detached, and 10,000 cells were applied to wells of an electrosensing plate coated with 10 μg/ml FN. The device was operated with RT-CES SP software. The cell index represents the extent of cell adhesion. The *bars* represent the S.D. *C*, cell migration toward FN was determined using the Transwell assay as described under "Experimental Procedures." Cells that migrated were stained with 0.5% crystal violet and counted under a microscope. The *bars* represent the S.D. A representative example is shown in the *right panel*. *D*, subconfluent cells were detached and labeled with primary antibody (mouse anti-human VLA5 antibody, HA5) for 30 min on ice. The labeled cells were washed with ice-cold phosphate-buffered saline and then incubated with Alexa Fluor 647 goat anti-mouse IgG for 30 min on ice. The expression levels of α5 integrin on the cell surface were measured using a FACSCalibur instrument (BD Biosciences). Negative controls were not treated with the primary antibody but underwent all other procedures.

formed fluorescence-activated cell sorting analysis using anti α5β1 integrin antibody. As shown in Fig. 3*D*, there were no significant differences in the levels of cell surface expression among the three cell types, indicating that the functional alterations shown in Fig. 3 were due to *N*-glycosylation of the integrin modified by GnT-III or GnT-V.

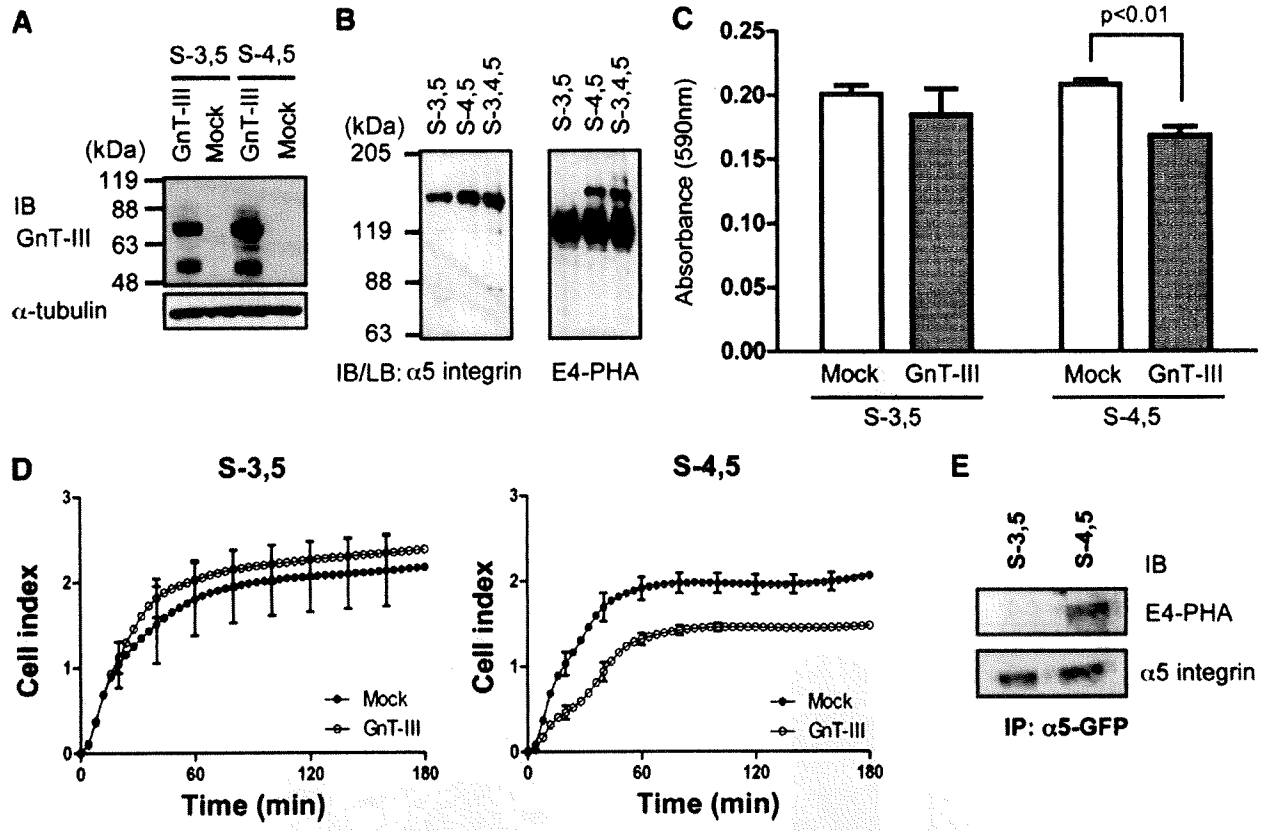

FIGURE 4. **Comparison of effects of GnT-III on *N*-glycosylation and cell adhesion between S-3,5 and S-4,5 mutants.** *A*, GnT-III was expressed in S-3,5 and S-4,5 mutants, and stable expression cells were established as described under "Experimental Procedures." The expression levels of GnT-III were detected with an antibody against GnT-III. *B*, confluent cells were lysed and then subjected to immunoprecipitation (*IP*) using an agarose-conjugated antibody against GFP. The immunoprecipitates were subjected to 6.0% SDS-PAGE under non-reducing conditions, blotted, probed with E4-PHA (*right panel*), and then reprobed with antibody against the α5 subunit (*left panel*). *C*, the cell adhesion assay was carried out as described above (Fig. 3*A*). The *bars* represent the S.D. *D*, the cell adhesion kinetics assay using the RT-CES system was the same as that described in Fig. 3*B*. Subconfluent cells were detached, and 40,000 cells were added to the wells. The cell index reflects the extent of cell adhesion. The *bars* represent the S.D. *E*, the S-3,5 and S-4,5 expression vectors were transfected into HeLa cells using the Phoenix retrovirus system and selected with puromycin as described under "Experimental Procedures." The cells were lysed, and the cell lysates (2 mg) were then subjected to immunoprecipitation using an agarose-conjugated antibody against GFP. The immunoprecipitates were subjected to 6.0% SDS-PAGE under non-reducing conditions, blotted, probed with E4-PHA (*upper panel*), and then reprobed with antibody against the α5 subunit (*lower panel*). *IB*, immunoblot; *LB*, lectin blot.

GnT-III Selectively Modifies N-Glycosylation Site-4 on the α5 Subunit—As described above, the characteristics of the S-3,4,5 mutant are similar to those of wild-type α5. We therefore determined whether GnT-III could specifically modify the *N*-glycosylation site among site-3, site-4, and site-5. Because the *N*-glycosylation site-5 is essential for its expression on the cell surface, the mutant did not exhibit biological function such as cell adhesion (24). Thus, we chose the S-3,5 and S-4,5 mutants for use in further studies.

First GnT-III was overexpressed in both transfectants. The expression levels of GnT-III were almost the same in S-3,5 as in S-4,5 transfectants, which were examined by Western blot using anti-GnT-III antibody (Fig. 4*A*). It is of particular interest that the mutant S-4,5, but not the S-3,5 mutant, was clearly detected using E4-PHA lectin blot. The intensity of the lectin staining was comparable to that of S-3,4,5 (Fig. 4*B*). These results, taken together, suggest that site-3 may not be modified by GnT-III. Because introduction of bisecting GlcNAc into the α5 subunit down-regulates cell adhesion as described above, we checked whether this phenomenon occurred in these mutants. Overexpression of GnT-III in S-4,5 cells consistently inhibited cell adhesion on FN, whereas the inhibition of cell adhesion was not observed in S-3,5 cells overexpressing GnT-III (Fig. 4, *C* and *D*). It should be noted that cell adhesion activities of the S-3,5 mutant was similar to that of S-4,5 mutant because CHO-B2 cells do not express enough endogenous GnT-III to modify integrin as shown in Fig. 2.

To confirm whether the site-specific modification also happens in endogenous conditions, we introduced the S-3,5 and S-4,5 mutants into HeLa cells that express a relatively higher level of endogenous GnT-III to examine the products of GnT-III as confirmed by E4-PHA lectin blot. Consistent with the results of overexpressing GnT-III, the E4-PHA lectin staining was clearly detected in S-4,5 but not in S-3,5 α5 subunit transfectants (Fig. 4*E*). Taken together, these results strongly suggest that GnT-III may specifically modify site-4 on the α5 subunit, which down-regulates its biological functions.

To further elucidate the importance of site-4 for GnT-III modification, we compared the E4-PHA staining patterns of WT with single mutants, such as site-3 (D-3) or site-4 (D-4), as shown in Fig. 1*A*. These three expression plasmids were co-transfected with GnT-III, and the α5 integrins were immuno-

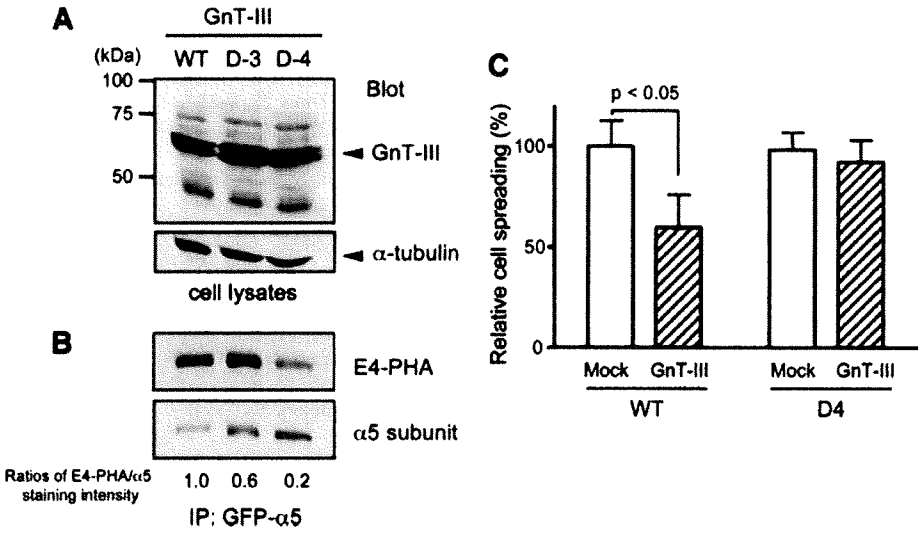

FIGURE 5. **Comparison of GnT-III modification among wild-type (WT), site-3 (D-3) and site-4 (D-4) deletion mutants.** GnT-III was expressed in WT and D-3 and D-4 deletion mutants, and stable expression cells were established as described under "Experimental Procedures." The expression levels of the GnT-III and GnT-III products in total cell lysates were detected with antibodies against GnT-III (*A, upper panel*) and α-tubulin to ensure equal loading (*A, lower panel*), respectively. *B*, the cell lysates were subjected to immunoprecipitation (*IP*) using an agarose-conjugated antibody against GFP. The immunoprecipitates were separated by 6.0% SDS-PAGE under non-reducing conditions. The membrane blot was probed with E4-PHA lectin and then was reprobed with an antibody against the α5 subunit. The ratio of E4-PHA to total α5 staining in WT cells was set equal to 1.0. *C*, the percentages of spread cells were quantified and expressed as the mean ± S.D. from three independent experiments. The *bars* represent S.D. The rounded cells were not considered as spread cells. The ratio of spread cells *versus* total cells (~300 cells) of WT transfectants was set as 100.

precipitated using an anti-GFP antibody. As shown in Fig. 5*A*, there were no significant differences in the GnT-III expression levels among the three transfectants. The intensity of E4-PHA staining in D-3 cells was less than that in WT cells, but they were comparable. However, the intensity of E4-PHA staining in D-4 cells was substantially less than that in WT or D-3 cells (Fig. 5*B*). The ratios of E4-PHA staining *versus* total α5 staining purified from WT, D-3, and D-4 cells were 1.0, 0.6, and 0.2, respectively. Furthermore to directly examine the effects of GnT-III on site-4 for cell adhesion, we compared cell spreading of the D-4 mutant with WT of α5 integrin. As expected, GnT-III significantly down-regulated cell spreading on FN in WT transfectants, whereas the deletion of site-4 abolished the suppression of cell spread induced by GnT-III in D-4 transfectants (Fig. 5*C*). Taken together, these results clearly show that *N*-glycosylation of site-4 is critical and effective for GnT-III modification.

DISCUSSION

In the present study, we intensively investigated the effects of *N*-glycosylation on the β-propeller of the integrin α5 subunit on its biological functions such as cell adhesion and cell migration and found that site-4 is essential and effective for GnT-III modification among 14 potential *N*-glycosylation sites. To our knowledge, this is the first report to clearly demonstrate that a glycosyltransferase of *N*-glycosylation can specifically modify an *N*-glycosylation site among multiple potential sites and effectively regulate its biological functions.

Integrins can be activated by inside-out signaling mechanisms that trigger global conformational changes, which ultimately modulate integrin-ligand affinity. It is apparent that integrin activity can be regulated by other mechanisms, such as posttranscriptional modification, *N*-glycosylation. Altered integrin glycosylation has been associated with tumorigenesis, autoimmune disease, chronic inflammation, and cell adhesion events (11, 30). In particular, *N*-glycosylation of the integrin α5 and β1 subunits appears to be important for both structure and function. It has been reported that *N*-glycosylation of both the α5 and β1 subunits is necessary for α5β1 heterodimerization and its binding to FN. Moreover changes in integrin glycan composition, resulting from forced expression of selected glycosyltransferases, *i.e.* "remodeling," reportedly modulate integrin functions as described above. However, most of these earlier studies examined only total changes without individual information. Therefore, the exact molecular mechanisms by which *N*-glycosylation of site(s) or glycan(s) occurs remain unknown. Recently we used site-directed mutagenesis to determine that *N*-glycosylation site-5 on the β-propeller plays an important role in the assembly of the integrin for its expression on the cell surface (24). These observations prompted us to determine whether there are specific *N*-glycosylation sites that regulate its biological functions. Here we clearly showed that site-4 is a key *N*-glycosylation site for the biological function of α5 subunit that is effectively modified by GnT-III. Taken together these results indicate that individual *N*-glycosylation sites may have unique functions.

Although the molecular mechanism by which bisecting GlcNAc is introduced into site-4, inhibiting its biological function, remain unknown, we speculate that the effect of altered glycosylation of site-4 may be related to conformational changes in the key functional regions of the β-propeller domain of the α5 subunit that are critical for integrin activation. In fact, the β-propeller domain has been postulated to be required for effective interaction between α5β1 integrin and its ligand (31). In contrast, the crystal structure of integrin αVβ3 has been successfully determined, and the main contact between the αV and β3 subunits is the β-propeller on the α and A domain on β3 with hydrophobic, ionic, and mixed contacts (32, 33). Because the α5 subunit has 47% homology to αV, Mould *et al.* (34) made a homologous modeling structure of α5β1. Based on the model, the α5 subunit seems to be surrounded by *N*-glycans, explaining the dissociation of the αβ heterodimer that occurs when α5β1 is deglycosylated by treatment with peptide-*N*-glycosidase F or removal of *N*-glycans on the β-propeller. Very recently, Liu *et al.* (35) used a molecular modeling approach to study the effects of altered glycosylation on the I-like domain of

the β1 subunit, which is the partner of the β-propeller of the α subunit. These researchers found that α2,6 sialic acid affected the interactions between *N*-glycans and the I-like domain, which in turn altered the accessibility of the loop that determines specificity of ligand binding. In fact, the remodeling of *N*-glycans by GnT-III affects either the branching formations catalyzed by GnT-V and GnT-IV or the sialylation on the terminus of the *N*-glycans (11, 36). Therefore, a possible mechanism by which *N*-glycans are involved in the αβ interaction or conformational arrangement is that an unknown lectin domain may exist on the α or β subunit. The lectin domain of αMβ2 integrin is associated with GlcNAc on the non-reducing terminus of sugar chains on platelets, facilitating their phagocytosis (37, 38). These studies further support the observation that modification of bisecting GlcNAc on site-4 of the β-propeller may be critical for the regulation of its biological functions, which may shed light on the structural studies.

It is of interest to understand why GnT-III specifically and effectively modifies site-4 of the 14 putative *N*-glycosylation sites. There is currently no detailed information available regarding this observation, but several explanations have been proposed. First, *N*-glycosylation occurs on site-4 because it provides the easiest access for GnT-III. Because the integrin α5 crystal structure is currently unavailable this hypothesis cannot be proven. Second, GnT-III may associate with some other molecules, which define the specificities for protein or peptide substrates. Reportedly protein *O*-mannosyltransferase 1 (POMT1) and its homolog POMT2 are responsible for catalyzing the first step in *O*-mannosyl glycan, which is important for muscle and brain development (39). Interestingly Manya *et al.* (40) reported that formation of a POMT1-POMT2 complex is essential for POMT activity. Only two peptides derived from the mucin domain of α-dystroglycan are highly *O*-mannosylated by POMT, but no *O*-mannosylation occurs in mucin tandem repeat peptides (41). Similarly complex formation is also important for T-synthase (core 1 β1,3-galactosyl transferase) activity. Ju *et al.* (42, 43) reported that Tn syndrome, a rare autoimmune disease, in which subpopulations of blood cells of all lineages carry an incompletely glycosylated membrane glycoprotein, known as the Tn antigen, is associated with a somatic mutation in *Cosmc*, a gene on the X chromosome that encodes a molecular "chaperone" that is required for the proper folding and hence full activity of T-synthase. Indeed it has been reported that caveolin-1 may co-localize with GnT-III to regulate its localization and activity (44). Those results, taken together, suggest that glycosyltransferase complex formation may play a crucial role in determination of both activity and substrate specificity. The detailed molecular mechanism requires further study.

This study specifically focused on *N*-glycosylation of the integrin α5 subunit. To fully understand the effects of the *N*-glycans on integrin structure and function, it will be necessary, in future studies, to investigate the interaction of glycans with glycans or peptides of integrin. The current study also has implications for engineering α5 that contains the glycans necessary for its activation that may facilitate the study of its crystal structure.

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